Important Late-Stage Symbiotic Role of the Sinorhizobium Meliloti Exopolysaccharide Succinoglycan

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Important late stage symbiotic role of the *Sinorhizobium meliloti* exopolysaccharide succinoglycan

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Running title: Protective effect of *S. meliloti* succinoglycan

Abstract

Sinorhizobium meliloti enters into a beneficial symbiotic interaction with species of *Medicago* legumes. Bacterial exopolysaccharides play critical signaling roles in infection thread initiation and growth during the early stages of root nodule formation. After endocytosis of *S. meliloti* by plant cells in the developing nodule, plant-derived nodule-specific cysteine-rich (NCR) peptides mediate terminal differentiation of the bacteria into nitrogen-fixing bacteroids. Previous transcriptional studies have shown that the intensively studied cationic peptide NCR247 induces expression of the *exo* genes that encode the proteins required for succinoglycan biosynthesis. In addition, genetic studies have shown that some *exo* mutants exhibit increased sensitivity to NCR247's antimicrobial action. We therefore investigated whether the *S. meliloti* symbiotically active exopolysaccharide, succinoglycan can protect *S. meliloti* against the antimicrobial activity of NCR247. We discovered that high molecular weight forms of succinoglycan have the ability to protect from the antimicrobial action of the NCR247 peptide, but that low molecular weight forms of wild-type succinoglycan do not. The protective function of high molecular weight succinoglycan occurs via direct molecular interactions between anionic succinoglycan and the cationic NCR247 peptide but this interaction is not chiral. Taken together, our observations suggest that *S. meliloti* exopolysaccharides may not only be critical during early stage nodule invasion, but are also up-regulated again at a late stage of symbiosis to protect bacteria against the bactericidal action of cationic NCR peptides. Our findings are an important step forward in fully understanding the complete set of exopolysaccharide functions during the legume symbiosis.

Importance

Symbiotic interactions between Rhizobia and legumes are economically important for global food production. The legume symbiosis also is a major part of the global nitrogen cycle and is an ideal model system to study host-microbe interactions. Signaling between legumes and Rhizobia is essential to establish symbiosis and understanding these signals is a major goal in the field. Exopolysaccharides are important in the symbiotic context because they are essential signaling molecules during early stage symbiosis. In this study we provide evidence suggesting that the *Sinorhizobium meliloti* exopolysaccharide, succinoglycan also protects the bacteria against the antimicrobial action of essential late stage symbiosis plant peptides.

Introduction

The nitrogen-fixing bacterium *Sinorhizobium meliloti* can enter into a mutually beneficial symbiosis with *Medicago* plant species. Under nitrogen-limiting environmental conditions, *Medicago* plants secrete aromatic flavonoid compounds into the surrounding soil, which signal *S. meliloti* bacteria to synthesize and secrete lipochitooligosaccharide Nod factor. Nod factor induces plant root hair curling to trap surrounding bacteria. Subsequently, further signaling between the plant and the bacteria leads to the initiation of tubular structures called infection threads that elongate in the root hair toward cortical root cell layers. These infection threads support bacterial propagation and invasion into the developing nodule, eventually leading bacteria to specialized host cells that use an endocytic-like process to internalize bacteria. Intracellular *S. meliloti* bacteria are contained within a host-derived membrane compartment, called a symbiosome, and host signals subsequently cause these internalized bacteria to differentiate into nitrogen-fixing bacteroids. *S. meliloti* bacteroids supply fixed nitrogen in the form of ammonia (NH_3) to the plant-host in exchange for fixed carbon in form of dicarboxylic acids (1).

Much of our understanding of the signaling molecules and pathways required for the nitrogen-fixing symbiosis stem from genetic studies in *S. meliloti* and the host plants *Medicago truncatula* and *M. sativa*. Research by our lab and others has shown that *S. meliloti* exopolysccharides are a key signaling molecule for infection thread initiation and growth (2-5). *S. meliloti* is capable of synthesizing the exopolysaccharides succinoglycan and galactoglucan, and both polysaccharides can independently support symbiosis with host plants. Succinoglycan is a polymer of octasaccharide subunits, each of which is composed of one galactose and seven glucose residues modified with acetyl, succinyl, and pyruvyl groups (6). Galactoglucan is a polymer of disaccharide subunits, each of which is composed of a glucose and galactose residue, modified with an acetyl and a pyruvyl group, respectively (7). *S. meliloti* produces and secretes two forms of succinoglycan, a high molecular weight (HMW) form composed of hundreds of octasaccharide subunits (8) and a low molecular weight (LMW) form composed of 1-3 octasaccharide

subunits (9). Of these *S. meliloti* polysaccharides, succinoglycan is the best understood in terms of its structure and functions during the legume symbiosis. *S. meliloti* bacteria that harbor mutations abolishing succinoglycan biosynthesis, modification, or polymerization initiate the legume symbiosis and promote root hair curling, but either fail to induce the host to form an infection thread through which plant tissue invasion is achieved or to support continued growth of an infection thread, if one is initiated (3). Results of previous work suggest that *S. meliloti* bacteria that lack functional ExoY, the phosphoglycosyltransferase that initiates succinoglycan biosynthesis, elicit stronger expression of plant defense genes than wild-type bacteria, suggesting one potential mode of action of succinoglycan is to dampen the response of the plant immune system (10). This phenotype led to the hypothesis that one function of succinoglycan is to serve as a signaling molecule to the host plant. Recent research by Kawaharada *et al*. (11, 12) confirmed this hypothesis by showing that a host plant receptor selectively binds to bacterial exopolysaccharides, and that this recognition by the bacterial exopolysaccharide receptor is necessary for infection thread growth and bacterial invasion across the epidermal cell layers.

Work in the host plant *M. truncatula* identified signaling factors that *S. meliloti* encounters after entering host cells, inside the symbiosomes. These signals are a family of more than 600 nodule-specific cysteine-rich (NCR) peptides that are essential for differentiation of bacteria into nitrogen fixing bacteroids (13-16). NCR peptides show high diversity in amino acid sequence, length and charge but have conserved patterns of either four or six cysteines (17). Recently, two NCR peptides, namely NCR211 (18) and NCR169 (19) have been shown to be essential for the *Sinorhizobium-Medicago* symbiosis. One NCR peptide that has been extensively studied for its physiological activities on *S. meliloti* is the cationic NCR247 peptide. NCR247 possesses antimicrobial activity at relatively high concentrations (20 μM) (13, 20). In contrast, at a sub-lethal dose (4 μM) NCR247 induces massive transcriptional changes (about 20% of annotated genes) in cell cycle synchronized bacterial cultures, inhibits cell division, and inhibits translation (15, 21). This group of genes include all genes involved in the synthesis of succinoglycan.

NCR247 has the sequence, RNGCIVDPRCPYQQCRRPLYCRRR, which includes four cysteines, six arginines and also some hydrophobic residues. When NCR247 is oxidized, the presence of four cysteines allow for the formation of three distinct, oxidized regioisomers with different disulfide connectivities. Each regioisomer has its unique effects on the action of NCR247 affecting its various physiological actions (21). The disulfide connectivity inside *Medicago* root nodules has not yet been established for any NCR peptide.

The exopolysaccharide succinoglycan is known to be important for early symbiosis. However, recently published studies suggest late stage symbiotic functions of succinoglycan. Interestingly, the many genes whose transcription is modulated by NCR247 treatment include the ExoS-ChvI regulon, which comprises the genes involved in exopolysaccharide synthesis, export, and polymerization (15, 22). Furthermore, a recent genomic, sequencing based, screen (Tn-seq) for transposon mutations that sensitize to or protect *S. meliloti* against NCR247 antimicrobial activity has found that among those 78 genes affecting NCR247 sensitivity are some of the very same genes induced by NCR247 treatment (15, 23). Namely, transposon insertions in genes *exoW*, *exoV*, *exoT* and *exoQ,* that are part of the final stages of succinoglycan biosynthesis resulted in significant loss in competitiveness during NCR247 treatment (23). Therefore, NCR247 induced expression of these genes and an increase in exopolysaccharide expression may be required for resistance to the bactericidal effects of NCR247 during bacteroid differentiation (23). Additional evidence for late stage functions of succinoglycan can be hypothesized based on published transcriptome data derived from laser-disected nodule sections, which show that a number of genes encoding succinoglycan biosynthesis proteins are up regulated later during symbiosis (Fig. S1A) (24). It remains to be determined why genes that encode for exopolysaccharide biosynthesis genes are up regulated past the initial stage of symbiosis, for which they have been shown to be essential.

In this study, we investigated whether a second mode of action by which *S. meliloti* exopolysaccharides might exert their biological effects is by modulating the antimicrobial activity of NCR peptides later during the symbiosis. To investigate this issue, we treated *S. meliloti* succinoglycan

biosynthesis gain-of-function and loss-of-function mutants with a lethal dose of the model peptide NCR247 and demonstrated that the production of succinoglycan results in higher resistance to the antimicrobial action of NCR247. Using two independent approaches for peptide-polysaccharide interactions, we obtained evidence that succinoglycan and NCR247 directly interact, and that this direct interaction is affected by the presence of both anionic modifications, the pyruvyl and the succinyl residues and the non-charged acetyl modification. We provide evidence that this interaction is, at least partly, of ionic nature. Thus, our results suggest that, in addition to serving as signaling molecules in incipient infection threads, the exopolysaccharide succinoglycan may also provide a level of protection to *S. meliloti* cells when they are exposed to NCR247 and similar NCR peptides in the symbiosomes of host cells.

Results

Strains that overproduce succinoglycan have enhanced resistance to NCR247 antimicrobial activity. Previously, we showed that mutations that eliminate the final processing steps of succinoglycan production sensitize *S. meliloti* to the antimicrobial activity of NCR247, and we hypothesized that succinoglycan production may provide protection against NCR247 (23). An alternative possibility is that mutations in genes that affect the final processing steps of succinoglycan may stress and weaken cellular structures, increasing the cells' sensitivity to antimicrobial peptides like NCR247 that target the cell membrane. To begin testing the hypothesis that succinoglycan production is protective against NCR247, we treated strains that overproduce succinoglycan and parental wild-type strains with a dose of NCR247 that is lethal to the wild-type *S. meliloti* strain *Sm*1021 and monitored bacterial survival hourly over a five-hour period of time during treatment. In this experiment, we used *Sm*1021 derivatives carrying either the *chvI*^{D52E} (25) or *exoS96* (26) mutation, which both result in the hyper-activation of the ExoS-ChvI two-component system. The ExoS-ChvI two component system regulates a suite of genes, including those for

158 succinoglycan biosynthesis (25), and expression of the ExoS-ChvI regulon is stimulated by bacteriostatic
159 levels of NCR247 (15). We found that strains bearing either the *chvI*^{D52E} (Fig. 1A, empty squares) or *exoS96*
160 (Fig. 1B, empty squares) alleles exhibited a significant increase in resistance to NCR247 relative to their
161 parental wild-type or *exoY* mutant control strains (empty circles and empty triangles, respectively) that
162 possess similar antimicrobial sensitivity to NCR247 under our experimental conditions (23). The protective
163 effect of the *chvI*^{D52E} and *exoS96* alleles was dependent on succinoglycan production, as loss-of-function
164 mutations in either *exoY* (Fig. 1A, empty diamonds) or *exoA* (Fig. 1B, empty diamonds), which encode the
165 UDP-galactose-undecaprenyl-phosphate galactose-I-phosphate transferase and a glycosyltransferase,
166 respectively, that initiate succinoglycan production and therefore are essential for succinoglycan
167 production (22), significantly increased NCR247 sensitivity, compared to the *S. meliloti* 1021 derivatives
168 with the *chvI*^{D52E} or *exoS96* alleles (Fig. 1 A and B). These observations show that constitutive activation of
169 the ExoS-ChvI regulon enhances the resistance of *S. meliloti* to NCR247 and that this increased resistance
170 is dependent on a functional succinoglycan biosynthesis pathway.

171 We also assayed the sensitivity of bacteria carrying the partial loss of function allele *ChvI*^{K214T},
172 which significantly depresses expression of the ExoS-ChvI regulon including succinoglycan biosynthesis
173 genes (27). Consistent with the above results, we found that the *ChvI*^{K214T} (Fig. 1C, empty triangles) strain
174 exhibited enhanced sensitivity to NCR247 antimicrobial activity relative to wild-type cells (Fig. 1C, empty
175 circles).

176 **Exogenous addition of succinoglycan protects *S. meliloti* against the antimicrobial activity of**
177 **NCR247.** We investigated whether exogenous addition of succinoglycan could protect *S. meliloti* that was
178 simultaneously treated with a bactericidal dose of NCR247. We lyophilized the supernatant of cultures of
179 *exoS96* mutant cells, re-dissolved it in water, determined the polysaccharide concentration using
180 anthrone-sulfuric acid assays and then added defined concentrations of polysaccharide to *S. meliloti* wild-
181 type cells along with NCR247. We found that 10 μg ml^{-1} of exogenous succinoglycan increased the survival

of NCR247-treated cells by about ten-fold relative to cells treated with NCR247 alone (Fig. 2A). Interestingly, we also observed a similar level of protection afforded to NCR247-treated *S. meliloti* with succinoglycan at 50 μg ml^{-1} (Fig. 2A). Thus, these findings indicate that the presence of succinoglycan can provide protection to *S. meliloti* from the antimicrobial effects of NCR247.

HMW succinoglycan is essential for protection against the antimicrobial action of NCR247. *S. meliloti* secretes two forms of succinoglycan, a HMW form and a LMW form (9). We tested whether both, the HMW and LMW forms of succinoglycan could protect *S. meliloti* from the antimicrobial effects of NCR247. Using the supernatant of the succinoglycan overexpressing *exoS96* mutant strain, we separately precipitated the HMW and LMW fractions of succinoglycan as described previously (9) and then tested the effects of each fraction on the bactericidal action of NCR247. We found that addition of HMW succinoglycan (10 μg ml^{-1}) enhanced the survival of *S. meliloti* by an order of magnitude relative to cultures treated with NCR247 alone (Fig. 2B). In contrast, addition of the LMW form (10 μg ml^{-1}) had no effect on the survival of *S. meliloti* treated with NCR247 (Fig. 2B). Thus, the HMW form of succinoglycan is necessary to enhance the survival of *S. meliloti* treated with NCR247.

As an additional control we analyzed the supernatant of an *exoY* mutant after precipitation with three volumes of ethanol. As published recently (23), an *exoY* mutant that lacks the ability to synthesize succinoglycan does not show increased sensitivity to NCR247 (Fig. S2A). We collected fractions 20-35 after separating wild-type and *exoY* supernatant after ethanol precipitation on a BioGel P-6 column and assessed whether these preparations were able to provide protection against the antimicrobial action of NCR247 (refer to later sections for size exclusion column data). We found that the supernatant collected from an *exoY* mutant does not contain any significant amounts of polysaccharide that corresponds to HMW succinoglycan (Fig. 5 A and D). As expected, we found that the polysaccharide material collected from fractions 20-35 derived from wild-type supernatant did protect against NCR247 (Fig. S2) and that the material recovered from the corresponding fractions of an *exoY* mutant did not have the same effect on

NCR247 sensitivity (Fig. S2). This result confirms our findings that HMW succinoglycan protects against the antimicrobial action of NCR247.

Bacterial protection by HMW succinoglycan is not universal for cationic peptides. We found that HMW succinoglycan has protective properties during NCR247 treatment of *S. meliloti*. Therefore, we tested whether this protective function is a general property during challenge with cationic peptides or whether our findings are specific for NCR247. To test our hypothesis, we treated an *Sm*1021 wild-type strain with the antibiotic polymyxin B (28), the bee venom component (29) melittin, human alpha defensin 5 (HD5) (30), the human antimicrobial peptide LL-37 (31) and the bovine antimicrobial peptide fragment $Bac7_{1\text{-}35}$ (32). Our results indicate that the protective effects of succinoglycan against cationic peptides are not universal. Succinoglycan only protects against the antimicrobial effects of Bac7 to a similar degree as to NCR247 (Fig. 3A). These results suggest that there is something special about the effects of succinoglycan on the antimicrobial effects of NCR247.

Bacterial protection by HMW succinoglycan is not dependent on chiral interactions. One way to probe the nature of a molecular interaction is to change the chirality of one of the interacting biomolecules (32, 33). Therefore, we obtained and purified an all D-enantiomer of NCR247 that is made up of only D-amino acids and found its lethality to be indistinguishable from that of the L-enantiomer (Fig. 3B). To test whether succinoglycan also protects against the NCR247 D-enantiomer, we assayed and compared the degree to which exogenously added HMW succinoglycan antagonizes the L- and the D-enantiomers of NCR247 and found that HMW succinoglycan antagonized both NCR247 enantiomers to a similar extent (Fig. 3B). These observations suggest that succinoglycan antagonizes NCR247 largely through mechanisms that do not involve chiral interactions.

HMW succinoglycan directly interacts with NCR247. Since NCR247 carries six positive charges and, it seemed possible that the mechanism underlying succinoglycan attenuation of NCR247 bactericidal

activity is a direct interaction between anionic polysaccharide and the cationic peptide. To test this, we used two approaches with two different modified NCR247 peptides. First, we utilized size exclusion chromatography to investigate changes in the retention time of a FITC-labelled derivative of the reduced NCR247 peptide (FI-NCR247) (21) and the HMW fractions of wild-type succinoglycan. The bactericidal activity of FI-NCR247 is the same as of the unmodified, reduced NCR247 peptide (21). Initially, we used $CoCl_2$ to establish the column volume of a Bio-Gel P6 column (fractions 70-80, Fig. S3). Subsequently, we established that FI-NCR247 elutes slightly earlier in fractions 63-70 (Fig. 4, purple circles) by measuring the FITC fluorescence intensity for each elution fraction. *S. meliloti* HMW succinoglycan has been shown previously to run in the early eluting fractions that correspond to the void volume of a comparable column setup (9). We found that wild-type HMW eluted just after fraction 20 (Fig. 5A) and, therefore, we only analyzed elution fractions greater than 20 and smaller than 80 for the following experiments. When FI-NCR247 was co-loaded with HMW succinoglycan, much of the FITC fluorescence of FI-NCR247 eluted much earlier in a broad range from fraction 40 – 62 (Fig. 4, red squares) as compared to the purple curve representing only FI-NCR247. This shift in FI-NCR247 fluorescence is consistent with it binding directly to HMW succinoglycan but slowly coming off as the HMW succinoglycan progresses down the column. Analyzing the amount of polysaccharide content in each fraction using the anthrone-sulphuric acid assay showed that most of the HMW wild-type succinoglycan still eluted in fractions 20 to 30, when co-loaded with FI-NCR247 (Fig. 5B). We also determined that LMW succinoglycan eluted between HMW succinoglycan and FI-NCR247 in fractions 37 and 44 (Fig. 5C).

As a second approach to investigate the direct interactions between NCR247 and succinoglycan, we used Octet (ForteBio) bio-layer interferometry. This method differs in multiple ways from our size exclusion column experiments. This methodology is used to determine direct molecular interactions based on light interference patterns changed by molecular binding of an analyte molecule in solution to an immobilized, biosensor-tip bound ligand molecule. An increase in binding signal is the net difference

of binding and dissociation processes. Our experimental setup used streptavidin coated biosensor tips loaded with biotinylated NCR247 peptide (see Material and Methods for peptide details). An experiment using the ForteBio Octet platform for bio-layer interferometry involves multiple stages. After incubation of the biosensor tips in assay buffer, the first stage is ligand binding (Biotin-NCR247) to the streptavidin biosensor tips (Fig. 6A). Ligand binding is followed by analyte (HMW succinoglycan) association in assay buffer (Fig. 6A). Last, the association step is stopped by placing the biosensor tips into assay buffer without solubilized succinoglycan. Results shown in Fig. 6A represent two exemplary concentrations of NCR247 and 10 µg ml^{-1} of wild-type HMW succinoglycan. Based on preliminary experiments we decided to use NCR247 at a final concentration of 0.7 µM to load the biosensor tips for all the following experiments. All results shown for the following experiments focus on the association and dissociation stage of our binding studies with the data being Y-axis normalized to the beginning of the association stage.

We found that HMW wild-type succinoglycan rapidly binds to the immobilized biotinylated NCR247 peptide, as indicated by the rapidly increasing binding signal (Fig. 6B, dark blue). We did not observe significant reduction in the binding signal when the biosensors were placed back into assay buffer (Fig. 6B). These findings confirmed our results obtained with the size exclusion column (Fig. 4). As bio-layer interferometry only requires very small amounts of ligand and analyte, we were also able to test the interaction of NCR247 with LMW succinoglycan. We observed a significantly lower degree of binding as indicated by the nearly flat binding curve compared to the HMW succinoglycan curve (Fig. 6B, compare dark blue vs dark red).

The interaction between NCR247 and succinoglycan is salt dependent. To assess whether the observed interactions between HMW succinoglycan and NCR247 are ionic in nature, we conducted further experiments with a modified association buffer. To increase the ionic strength we added NaCl to a final concentration of 0.1 M. We found that 0.1 M NaCl in the association buffer drastically reduces the binding of HMW succinoglycan to biosensor bound NCR247 as compared to HMW succinoglycan without

277 additional NaCl (Fig. 6C). This observation indicates that the presence of NaCl ions competes with
278 succinoglycan molecules for binding to cationic NCR247. The small increase in binding signal after placing
279 the biosensor from the NaCl containing buffer back into assay buffer without NaCl may be the
280 consequence of differing optical properties between buffers that affect sensor reading in the presence of
281 HMW succinoglycan (Fig. 6C). For LMW succinoglycan, NaCl in the association buffer only had very little
282 effect on the low amount of binding that was observed (Fig. 6C). Next, we tested whether we can could
283 use NaCl to force dissociation of succinoglycan from the NCR247 loaded biosensor tips. When NCR247
284 loaded biosensors with bound HMW succinoglycan were placed into assay buffer containing 0.1 M NaCl,
285 the binding signal rapidly reduced to the level observed when succinoglycan was allowed to associate with
286 NCR247 in assay buffer supplemented with 0.1 M NaCl (Fig. 6D). Taken together, these observations
287 (Fig. 6C and D) suggest that the molecular basis of the interaction between NCR247 and succinoglycan is
288 at least partially ionic.

289 **Succinoglycan modifications succinyl, pyruvyl and acetyl affect the interaction between NCR247**
290 **and succinoglycan.** Succinoglycan is decorated with three well-characterized side modifications, a
291 succinyl, an acetyl and a pyruvyl residue. *S. meliloti* mutants that lack intact *exoH*, *exoZ* and *exoV* genes
292 lack these modifications, respectively. To further investigate the molecular characteristics of the
293 interaction between succinoglycan and NCR247 we decided to purify succinoglycan from these three
294 *S. meliloti* mutants. We collected the supernatant from these mutants and precipitated succinoglycan
295 with three volumes of ethanol.

296 Succinoglycan produced by an *exoH* mutant lacks the anionic succinyl modification (34). An *exoH*
297 mutant that has been well characterized predominantly produces HMW succinoglycan (8). Just like wild-
298 type HMW succinoglycan, HMW succinoglycan of an *exoH* mutant is able to protect against the
299 antimicrobial action of NCR247 (Fig. 7). Bio-layer interferometry assays show that *exoH* mutant
300 succinoglycan displays a substantially increased binding rate and a higher degree of binding as compared

301 to wild-type HMW succinoglycan (Fig. S4A and Fig. 8A). We also observed that the addition of NaCl to the
302 assay buffer after the NCR247-*exoH* succinoglycan association results in a slower reduction in the binding
303 signal from the NCR247 coated biosensor tip as compared to wild-type succinoglycan (Fig. 8A). This
304 indicates that NCR247 forms a stronger association with *exoH* succinoglycan than wild-type succinoglycan
305 (Fig. 8A) and that the loss of this particular anionic succinoglycan modification results in an increase of
306 NCR247-succinoglycan binding. It is interesting that, despite its negative charge, the succinyl group seems
307 to impair the ability of succinoglycan to interact with NCR247 and that the improved binding observed in
308 the absence of the succinyl modification might involve hydrophobic interactions rather than ionic
309 interactions.

310 To assess whether the improved binding of the HMW succinoglycan obtained from the *exoH*
311 mutant might be due to hydrophobic interactions, potentially involving the two methyl groups on the
312 acetyl and pyruvyl groups respectively, we performed Octet binding assays in the presence of ethanol. We
313 found that the ability of wild-type HMW succinoglycan to bind NCR247 is not affected by ethanol (Fig. 8B,
314 association stage). The wild-type HMW samples, incubated in the presence of ethanol showed a higher
315 rate of dissociation when the sensor tips were placed into ammonium acetate buffer supplemented with
316 NaCl (Fig. 8B, dissociation stage). In contrast, NCR247 binding to succinoglycan precipitated from *exoH*
317 mutant supernatant was drastically reduced in the presence of ethanol and was dependent on the ethanol
318 concentration (Fig. 8B). These findings indicate that hydrophobic interactions between NCR247 and
319 succinoglycan play a stronger role when the succinyl modification is missing from succinoglycan.

320 Mutation of the *exoZ* gene results in the secretion of succinoglycan lacking the non-charged acetyl
321 modification (35). Functional ExoZ is not essential for a successful symbiosis (36). We precipitated HMW
322 succinoglycan and then LMW succinoglycan with an additional seven volumes of ethanol from the
323 supernatant of this mutant. We found that *exoZ* HMW succinoglycan is able to protect *S. meliloti* against
324 the antimicrobial action of NCR247 (Fig. 7). Our results using bio-layer interferometry show that HMW

and LMW succinoglycan derived from an *exoZ* mutant bind NCR247 more efficiently than the corresponding samples prepared from wild-type supernatant (Fig. S4B and Fig. 8A). Interestingly, as was the case for the succinyl modification, the effect of the acetyl modification appears to be to attenuate interaction between NCR247 and succinoglycan.

Finally, we examined whether succinoglycan from an *exoV* mutant that lacks pyruvyl modified succinoglycan is able to bind to NCR247. The pyruvyl modification of succinoglycan was hypothesized to be important for the formation of HMW succinoglycan, suggesting that an *exoV* mutant would be unable to produce significant amounts of HMW succinoglycan (37). We observed that the succinoglycan in the supernatant of the *exoV* mutant that precipitated after the addition of three volumes of ethanol contained a small proportion of HMW succinoglycan and a correspondingly larger amount of LMW molecules as compared to the wild-type three volume of ethanol preparation (compare Fig. 5A and Fig. 5E). This preparation does not protect from the antimicrobial activity of NCR247 (Fig. 7).

We purified fractions 37 to 47 that presumably contain LMW succinoglycan from the *exoV* mutant (Fig. 5E). We found that the succinoglycan from these fractions bound less well than HMW succinoglycan from wild type *S. meliloti,* but showed the same NCR247 binding profile as the *exoV* fraction precipitated with three volumes of ethanol (Fig. S4C) and the LMW succinoglycan from an *exoZ* mutant (Fig. 8A). We were unable to purify enough material for our experiments from the HMW peak (Fig. 5E). These findings confirm that the lack of the anionic pyruvyl modification interferes with the polymerization of succinoglycan subunits in HMW material (37), but also permits the LMW material to interact with NCR247 to some degree. However, this effect does not seem to be great enough to result in protection from the antimicrobial action of NCR247 (Fig. 7).

Discussion

Previously, our lab reported that the intensively studied NCR247 peptide induces global transcriptional changes in *S. meliloti* at a sub-lethal concentration (15). The genes whose expression was increased included those involved in the biosynthesis of succinoglycan. Furthermore, our research also identified several examples of genes required to synthesize succinoglycan that are important to protect cells from the antimicrobial effects of NCR247 (23). These observations suggest that NCR peptides up-regulate expression of succinoglycan biosynthesis genes and that succinoglycan then exerts a protective role that is potentially important for symbiosis. Therefore, we asked whether *S. meliloti* succinoglycan is able to protect the bacteria from the antimicrobial activities of the cationic model peptide NCR247.

We provide data suggesting that succinoglycan can protect *S. meliloti* against the antimicrobial activities of NCR247. We also show that succinoglycan must be present in its HMW form to exert this protective function. Furthermore, we used two independent methodologies with two distinct, modified NCR247 peptides to provide evidence that the protective function of NCR247 is relayed by direct, molecular interactions between the cationic NCR247 peptide and the anionic HMW succinoglycan. Hence, in addition to its role in promoting and maintaining directed infection thread growth, our data suggest that *S. meliloti* exopolysaccharides may have an additional symbiotic function, namely interacting with cationic NCR peptides to reduce their potential bactericidal effects.

Succinoglycan is essential for *S. meliloti* to form a successful symbiosis with *Medicago* plant species by mediating directed and efficient infection thread growth inside root hairs (3). This conclusion is consistent with a previously published, comprehensive nodule laser-dissection gene expression study (24) that showed that all *exo* genes that are involved in succinoglycan biosynthesis are expressed at relatively high levels during nodule infection in early symbiotic stages and their expression then decreases (Fig. S1A) (24). However, eight of the succinoglycan biosynthesis genes are then re-expressed at significant

levels in the nitrogen fixation zone (ZIII), where also some of the commonly studied NCR peptides display elevated gene expression (Fig. S1B). These observations from nodule laser-dissection studies are consistent with the evidence presented here that succinoglycan may have a second, late-stage role during symbiosis, in which it helps modulate the potential toxicity of NCR peptides.

Our results suggest that it is the HMW form of succinoglycan that is required to protect against the antimicrobial action of NCR247, with the anionic charge of succinoglycan allowing it to function as a chelator to mask the cationic charges of NCR247. The observation that increasing the amount of HMW succinoglycan does not result in complete protection (Fig. S2) raises the possibility that succinoglycan may form a proximal protective layer around the bacteria that limits the antimicrobial effect of NCR247 on the bacteria. Our results also indicate that this effect is not stereospecific and thus is most likely due to electrostatic interactions with possible hydrophobic contributions as well. Another non-exclusionary possibility is that succinoglycan binds a yet to be identified bacterial receptor that is important for NCR247 activity, which results in buffering of NCR247 antimicrobial effects. The magnitude of this protective effect is not seen equally with all cationic antimicrobial peptides, we investigated (Fig. 3). Among the antimicrobial peptides we investigated, the protective effect of succinoglycan is strongest for NCR247 and the bovine proline-rich Bac7 peptides. Both of these peptides have been studied in the context of the symbiotically essential *S. meliloti* BacA protein (20, 38, 39). However, currently it is not known whether there is a functional connection between BacA and succinoglycan. To assess how broad this exopolysaccharide mediated protection is against NCR peptides, future work should examine NCR peptides of different charges and other antimicrobial peptides. LMW succinoglycan may not be able to form a dense enough, charged mesh to chelate the attacking NCR247. However, a recent study found LMW succinoglycan to be important for bacterial protection against acidic pH levels, similar to those encountered by bacteria inside symbiosomes (40).

It is interesting that loss of either the succinyl modification or the acetyl modification increased the interaction between HMW succinoglycan and NCR247. These observations raise the possibility that at least one biological role for these poorly understood exopolysaccharide modifications might be to tune their interactions with cationic NCR peptides so that lethality can be minimized while still allowing the peptides to exert their important effects during differentiation into bacteroids and during nitrogen fixation. Because loss of the pyruvyl modification also affects the efficiency of polymerization of succinoglycan octasaccharide subunits into polymer, it is more difficult assess its role. Nevertheless, our data are consistent with the anionic pyruvyl modification playing an important role in the interaction with the cationic NCR peptides.

It has been reported recently that NCR peptides, especially cationic NCR peptides, interact with the membranes of differentiating bacteria and mediate the formation of outer membrane vesicles or other changes, such as pore formation (41). By being secreted and forming a proximal protective layer around the bacteria, succinoglycan, with its anionic and non-charged modifications, may be acting as a regulator of NCR peptide biological activity on the bacterial membranes. This effect might be accomplished by direct binding to a select group of NCR peptides, possibly cationic ones. A delicate balance between hydrophilic and hydrophobic interactions with NCR peptides could be a key element for this interaction.

In conclusion, we propose that, in addition to its well-studied role in promoting infection thread initiation and growth early in symbiosis, succinoglycan plays a second independent role later in symbiosis. Our data suggest that HMW succinoglycan modulates the exposure of the internalized rhizobia to the cationic NCR peptides produced within the nodule to achieve the levels and balance of NCR peptides necessary for differentiation into bacteroids and bacteroid maintenance. An additional possible late stage role could be that LMW succinoglycan protects bacteria inside symbiosomes against pH stress (40).

Materials and Methods

Bacterial strains and reagents. All bacterial strains used in this study are listed in Table 1. *S. meliloti* strains were grown in Lysogeny broth supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ containing the desired antibiotics. Antibiotics were used at the following concentrations: 200 µg ml^{-1} streptomycin (Sm), 100 µg ml^{-1} Neomycin (Nm), 25 µg ml^{-1} Gentamycin (Gm), 50 µg ml^{-1} Hygromycin (Hyg).

Generation of double mutants. Double mutants *exoS*96/*exoA* and *chvI*^{D52E}/*exoY* were constructed using ϕM12 phage transduction by transducing the *exoY*::Tn5 and *exoA*::Tn233 mutations into the *chvI*^{D52E} and *exoS96* mutants, respectively using established methodology (42).

Synthesis of NCR247 peptide derivatives. The oxidized NCR247 (all regioisomers mix) peptide was prepared as previously described (21). The reduced L- and D-forms of NCR247 were commercially synthesized by Genscript (USA) and then purified by our lab to a purity of 99%.

The synthesis of Biotin-NCR247-Bpa_{red} was performed using standard Fmoc solid-phase peptide synthesis techniques in a custom-made 25-mL glass reaction vessel outfitted with a medium porosity frit and a "T"-bore for N_2 gas bubbling. The synthesis of Biotin-NCR247-Bpa_{red} was performed on a 0.01-mmol scale of Fmoc-Arg (Pbf) -Novasyn@TGA resin, at amino acid position 3, Gly was replaced with Lys (NCR247 [G3K-biotin]) and at position 20, Tyr was replaced with 4-benzoyl-L-phenylalanine (NCR247 [G3K-Biotin] [Y20Bpa]). Fmoc-Lys (Biotin)-OH and Nα-Fmoc-4-benzoyl-L-phenylalanine was coupled thrice using Nα-Fmoc-4-benzoyl-L-phenylalanine (10 equiv) and Fmoc-Lys (Biotin)-OH and dissolved in DMF (15 mL) containing HATU (10 equiv), HOAt (10 equiv) and DIPEA (20 equiv). After coupling of the N-terminal Arg residue, the resin was thoroughly washed with CH_2Cl_2, the Fmoc was removed using 20 mL of solution containing a TFA:1, 2-ethanedithiol (EDT):H2O:triisopropylsilane (TIS) (94:2.5:2.5:1) mixture for 10 min at 60 °C. The mixture was concentrated to a volume of ~5 mL by using a gentle stream of nitrogen. Ice-cold diethyl ether was added to the resulting concentrate, which resulted in precipitation of the crude

peptides, and the mixture was centrifuged in a Beckman Coulter GS-6R rotor (3,500 rpm × 15 min, 4 °C). The organic supernatant was decanted, and the pelleted precipitate was re-dissolved in 5 mL of 5% TFA in 3:1 H_2O/MeCN. Preparative RP-HPLC purification (10–60% B acetonitrile over 30 min,10 mL/min) afforded reduced Biotin-NCR247-Bpa_{red} (2.5 mg, 10% overall yield). The crude product was reduced by addition of 1 mM tris (2-carboxy) ethyl-1-phosphine (TCEP) in Tris-HCl, pH 8.0 and purified to obtain Biotin-NCR247-Bpa_{red}.

Peptide killing assays. The defined strains were grown to early exponential phase (OD_{600} between 0.1 and 0.3), washed three times with 0.85% saline (0.85g NaCl / 100 ml) and the re-suspended to a final OD_{600} of 0.1 in 3-(N-morpholino)propanesulfonic acid (MOPS)-buffered minimal medium (50 mM MOPS, 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, 19 mM glutamic acid, 0.004 mM biotin, pH 7.0) supplemented with 1% casamino acids (MOPS-GS + cas). Then the cells where treated with 20 µM of NCR247 peptide and the surviving cells were recovered by spotting 10 µl aliquots in triplicates on LBMC strep plates.

Purification of succinoglycan. Succinoglycan was purified and isolated using the exopolysaccharide overproducing *S. meliloti* mutant strain *exoS96* as previously described (9, 43). Cultures were grown in glutamate–D-mannitol–salts (GMS) medium (1x M9 media, 12 mM D-mannitol, 5 mM glutamic acid, 86 mM NaCl, 0.008 mM thiamine, 0.004 mM biotin, pH 7.0) supplemented with biotin and thiamine in a final volume of one liter containing Sm and Nm at 30 °C and 160 rpm shaking. The supernatant containing all exopolysaccharides was then collected (20,000 x g for 30 min) and secreted HMW and LMW succinoglycan was purified using a two-step ethanol precipitation as previously described (9). First, the supernatant was precipitated with the addition of three volumes of ethanol (3V fraction) (6000 x g for 30 min). Second, the supernatant of the first precipitation reaction was precipitated again by the addition of seven volumes of ethanol (7V fraction) (6000 x g for 30 min). After precipitation, HMW and LMW succinoglycan pellets were re-suspended in up to 5 ml of deionized H_2O and then lyophilized and re-suspended in deionized H_2O,

463 until used at working concentration in the defined experiments based their polysaccharide content as
464 determined by the anthrone-sulphuric acid assay (44).

465 **Base hydrolysis of succinoglycan.** Base hydrolysis of purified succinoglycan was performed as previously
466 described using 100 mM KOH at 20°C for 6 hours (9). Then the base-hydrolized (BH) succinoglycan solution
467 was neutralized using 1 M HCl. Then BH succinoglycan was precipitated using three volumes of ethanol,
468 followed by dialysis (Slide-A-Lyzer Dialysis cassettes, Thermo Scientific) against water for 48 hours with
469 regular water changes. Dialysis was followed by lyophilization and re-suspension of BH succinoglycan in
470 three ml of dH2O.

471 **Size exclusion chromatography.** Size exclusion chromatography of purified total succinoglycan was
472 performed as previously described with modifications on a column (1.5 by 90 cm) of Bio-Gel P-6 (fine
473 mesh Bio-Rad) (9). The column was pre-equilibrated with 0.1 M ammonium acetate (pH 5.0). For
474 purification of single succinoglycan fractions approx. 500 µg of purified succinoglycan were loaded onto
475 the column suspended in a final volume of 2 ml 0.1 M Ammonium Acetate buffer (pH 5.0). Whenever
476 mentioned, FI-NCR247 was loaded at a final concentration of 50 µg ml^{-1}. Fractions (1.6 ml) were collected
477 and the carbohydrate content of each fraction was determined by using the anthrone-sulphuric acid assay
478 (44). In brief, in a 96 well plate, 50 µl of size exclusion chromatography fraction were mixed with 150 µl of
479 glacial sulphuric acid containing anthrone (2 g l^{-1}). The samples were left at 4°C for 20 min, then kept at
480 70°C for one hour followed by incubation at room temperature for 20 min. Then the absorbance at 620 nm
481 was measured for each sample using a Tecan Spark 10M plate reader.

482 **Bio-layer interferometry.** Bio-layer interferometry was carried out using a ForteBio Octet® RED96 Bio-
483 Layer Interferometry following the manufacturer's instructions for a standard kinetic assay. Streptavidin
484 (SA) biosensor tips were incubated in 200 µl of assay buffer [0.1 M ammonium acetate (pH 5.0)], each for
485 60 s. Then biotinylated NCR247 was loaded onto each biosensor tip at the defined concentration until the
486 binding signal reached a value greater than 1.4. Biosensor tip loading was followed by incubation in assay

buffer for 60 s. Association between the ligand NCR247 and the analyte HMW succinoglycan (10 µg ml^{-1} in assay buffer) was observed over a timeframe of 300 s in assay buffer supplemented as indicate in the corresponding figures. For dissociation, to stop binding kinetics the biosensor tips were placed back into assay buffer not containing any succinoglycan with or without supplementation with 0.1 M NaCl for 120 s (as indicated in the corresponding figures). Data analysis was done using the Octet® Data Analyses 8.2 software.

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References

1. **Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC.** 2007. How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. Nat Rev Microbiol **5:**619-633.
2. **Battisti L, Lara JC, Leigh JA.** 1992. Specific oligosaccharide form of the *Rhizobium meliloti* exopolysaccharide promotes nodule invasion in alfalfa. Proc Natl Acad Sci U S A **89:**5625-5629.
3. **Pellock BJ, Cheng HP, Walker GC.** 2000. Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides. J Bacteriol **182:**4310-4318.
4. **Urzainqui A, Walker GC.** 1992. Exogenous suppression of the symbiotic deficiencies of *Rhizobium meliloti exo* mutants. J Bacteriol **174:**3403-3406.
5. **Mendis HC, Madzima TF, Queiroux C, Jones KM.** 2016. Function of Succinoglycan Polysaccharide in *Sinorhizobium meliloti* Host Plant Invasion Depends on Succinylation, Not Molecular Weight. mBio **7**.
6. **Leigh JA, Signer ER, Walker GC.** 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc Natl Acad Sci U S A **82:**6231-6235.
7. **Glazebrook J, Walker GC.** 1989. A novel exopolysaccharide can function in place of the Calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. Cell **56:**661-672.
8. **York GM, Walker GC.** 1998. The succinyl and acetyl modifications of succinoglycan influence susceptibility of succinoglycan to cleavage by the *Rhizobium meliloti* glycanases ExoK and ExsH. J Bacteriol **180:**4184-4191.
9. **Wang LX, Wang Y, Pellock B, Walker GC.** 1999. Structural characterization of the symbiotically important low-molecular-weight succinoglycan of *Sinorhizobium meliloti*. J Bacteriol **181:**6788-6796.
10. **Jones KM, Sharopova N, Lohar DP, Zhang JQ, VandenBosch KA, Walker GC.** 2008. Differential response of the plant *Medicago truncatula* to its symbiont *Sinorhizobium meliloti* or an exopolysaccharide-deficient mutant. Proc Natl Acad Sci U S A **105:**704-709.
11. **Kawaharada Y, Nielsen MW, Kelly S, James EK, Andersen KR, Rasmussen SR, Fuchtbauer W, Madsen LH, Heckmann AB, Radutoiu S, Stougaard J.** 2017. Differential regulation of the Epr3 receptor coordinates membrane-restricted rhizobial colonization of root nodule primordia. Nat Commun **8:**14534.
12. **Kawaharada Y, Kelly S, Nielsen MW, Hjuler CT, Gysel K, Muszynski A, Carlson RW, Thygesen MB, Sandal N, Asmussen MH, Vinther M, Andersen SU, Krusell L, Thirup S, Jensen KJ, Ronson CW, Blaise M, Radutoiu S, Stougaard J.** 2015. Receptor-mediated exopolysaccharide perception controls bacterial infection. Nature **523:**308-312.
13. **Van De Velde W, Zehirov G, Szatmari A, Debreczeny M, Ishihara H, Kevei Z, Farkas A, Mikulass K, Nagy A, Tiricz H, Satiat-Jeunemaître B, Alunni B, Bourge M, Kucho KI, Abe M, Kereszt A, Maroti G, Uchiumi T, Kondorosi E, Mergaert P.** 2010. Plant peptides govern terminal differentiation of bacteria in symbiosis. Science **327:**1122-1126.
14. **Farkas A, Maroti G, Durgo H, Gyorgypal Z, Lima RM, Medzihradszky KF, Kereszt A, Mergaert P, Kondorosi E.** 2014. *Medicago truncatula* symbiotic peptide NCR247 contributes to bacteroid differentiation through multiple mechanisms. Proc Natl Acad Sci U S A **111:**5183-5188.
15. **Penterman J, Abo RP, De Nisco NJ, Arnold MF, Longhi R, Zanda M, Walker GC.** 2014. Host plant peptides elicit a transcriptional response to control the *Sinorhizobium meliloti* cell cycle during symbiosis. Proc Natl Acad Sci U S A **111:**3561-3566.
16. **Maróti G, Downie JA, Kondorosi É.** 2015. Plant cysteine-rich peptides that inhibit pathogen growth and control rhizobial differentiation in legume nodules. Current Opinion in Plant Biology **26:**57-63.

17. **Mergaert P, Nikovics K, Kelemen Z, Maunoury N, Vaubert D, Kondorosi A, Kondorosi E.** 2003. A novel family in *Medicago truncatula* consisting of more than 300 nodule-specific genes coding for small, secreted polypeptides with conserved cysteine motifs. Plant Physiol **132:**161-173.
18. **Kim M, Chen Y, Xi J, Waters C, Chen R, Wang D.** 2015. An antimicrobial peptide essential for bacterial survival in the nitrogen-fixing symbiosis. Proc Natl Acad Sci U S A **112:**15238-15243.
19. **Horvath B, Domonkos A, Kereszt A, Szucs A, Abraham E, Ayaydin F, Boka K, Chen Y, Chen R, Murray JD, Udvardi MK, Kondorosi E, Kalo P.** 2015. Loss of the nodule-specific cysteine rich peptide, NCR169, abolishes symbiotic nitrogen fixation in the *Medicago truncatula dnf7* mutant. Proc Natl Acad Sci U S A **112:**15232-15237.
20. **Haag AF, Baloban M, Sani M, Kerscher B, Pierre O, Farkas A, Longhi R, Boncompagni E, Hérouart D, Dall'Angelo S, Kondorosi E, Zanda M, Mergaert P, Ferguson GP.** 2011. Protection of *Sinorhizobium* against Host Cysteine-Rich Antimicrobial Peptides Is Critical for Symbiosis. PLoS Biology **9:**e1001169.
21. **Shabab M, Arnold MF, Penterman J, Wommack AJ, Bocker HT, Price PA, Griffitts JS, Nolan EM, Walker GC.** 2016. Disulfide cross-linking influences symbiotic activities of nodule peptide NCR247. Proc Natl Acad Sci U S A **113:**10157-10162.
22. **Reuber TL, Walker GC.** 1993. Biosynthesis of succinoglycan, a symbiotically important exopolysaccharide of *Rhizobium meliloti*. Cell **74:**269-280.
23. **Arnold MFF, Shabab M, Penterman J, Boehme KL, Griffitts JS, Walker GC.** 2017. Genome-Wide Sensitivity Analysis of the Microsymbiont *Sinorhizobium meliloti* to Symbiotically Important, Defensin-Like Host Peptides. mBio **8**.
24. **Roux B, Rodde N, Jardinaud MF, Timmers T, Sauviac L, Cottret L, Carrere S, Sallet E, Courcelle E, Moreau S, Debelle F, Capela D, de Carvalho-Niebel F, Gouzy J, Bruand C, Gamas P.** 2014. An integrated analysis of plant and bacterial gene expression in symbiotic root nodules using laser capture microdissection coupled to RNA-seq. Plant J **77:**817-837.
25. **Chen EJ, Fisher RF, Perovich VM, Sabio EA, Long SR.** 2009. Identification of direct transcriptional target genes of ExoS/ChvI two-component signaling in *Sinorhizobium meliloti*. J Bacteriol **191:**6833-6842.
26. **Doherty D, Leigh JA, Glazebrook J, Walker GC.** 1988. *Rhizobium meliloti* mutants that overproduce the *R. meliloti* acidic calcofluor-binding exopolysaccharide. J Bacteriol **170:**4249-4256.
27. **Chen EJ, Sabio EA, Long SR.** 2008. The periplasmic regulator ExoR inhibits ExoS/ChvI two-component signalling in *Sinorhizobium meliloti*. Mol Microbiol **69:**1290-1303.
28. **Orwa JA, Govaerts C, Busson R, Roets E, Van Schepdael A, Hoogmartens J.** 2001. Isolation and structural characterization of polymyxin B components. J Chromatogr A **912:**369-373.
29. **Tosteson MT, Tosteson DC.** 1981. The sting. Melittin forms channels in lipid bilayers. Biophys J **36:**109-116.
30. **Ericksen B, Wu Z, Lu W, Lehrer RI.** 2005. Antibacterial activity and specificity of the six human α-defensins. Antimicrob Agents Chemother **49:**269-275.
31. **Gudmundsson GH, Agerberth B, Odeberg J, Bergman T, Olsson B, Salcedo R.** 1996. The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. Eur J Biochem **238:**325-332.
32. **Podda E, Benincasa M, Pacor S, Micali F, Mattiuzzo M, Gennaro R, Scocchi M.** 2006. Dual mode of action of Bac7, a proline-rich antibacterial peptide. Biochimica et Biophysica Acta - General Subjects **1760:**1732-1740.
33. **Wei G, de Leeuw E, Pazgier M, Yuan W, Zou G, Wang J, Ericksen B, Lu WY, Lehrer RI, Lu W.** 2009. Through the looking glass, mechanistic insights from enantiomeric human defensins. Journal of Biological Chemistry **284:**29180-29192.

34. **Leigh JA, Reed JW, Hanks JF, Hirsch AM, Walker GC.** 1987. *Rhizobium meliloti* mutants that fail to succinylate their Calcofluor-binding exopolysaccharide are defective in nodule invasion. Cell **51:**579-587.
35. **Reuber TL, Walker GC.** 1993. The acetyl substituent of succinoglycan is not necessary for alfalfa nodule invasion by *Rhizobium meliloti Rm*1021. Journal of Bacteriology **175:**3653-3655.
36. **Buendia AM, Enenkel B, Köplin R, Niehaus K, Arnold W, Pünier A.** 1991. The *Rhizobium meliloti exoZl exoB* fragment of megaplasmid 2: ExoB functions as a UDP-glucose 4-epimerase and ExoZ shows homology to NodX of *Rhizobium leguminosarum* biovar *viciae* strain TOM. Molecular Microbiology **5:**1519-1530.
37. **Glucksmann MA, Reuber TL, Walker GC.** 1993. Genes needed for the modification, polymerization, export, and processing of succinoglycan by *Rhizobium meliloti*: a model for succinoglycan biosynthesis. J Bacteriol **175:**7045-7055.
38. **Marlow VL, Haag AF, Kobayashi H, Fletcher V, Scocchi M, Walker GC, Ferguson GP.** 2009. Essential role for the BacA protein in the uptake of a truncated eukaryotic peptide in *Sinorhizobium meliloti*. J Bacteriol **191:**1519-1527.
39. **Wehmeier S, Arnold MF, Marlow VL, Aouida M, Myka KK, Fletcher V, Benincasa M, Scocchi M, Ramotar D, Ferguson GP.** 2010. Internalization of a thiazole-modified peptide in *Sinorhizobium meliloti* occurs by BacA-dependent and -independent mechanisms. Microbiology **156:**2702-2713.
40. **Hawkins JP, Geddes BA, Oresnik IJ.** 2017. Succinoglycan production contributes to acidic pH tolerance in *Sinorhizobium meliloti Rm*1021. Mol Plant Microbe Interact.
41. **Montiel J, Downie JA, Farkas A, Bihari P, Herczeg R, Balint B, Mergaert P, Kereszt A, Kondorosi E.** 2017. Morphotype of bacteroids in different legumes correlates with the number and type of symbiotic NCR peptides. Proc Natl Acad Sci U S A **114:**5041-5046.
42. **Finan TM, Hartweig E, LeMieux K, Bergman K, Walker GC, Signer ER.** 1984. General transduction in *Rhizobium meliloti*. J Bacteriol **159:**120-124.
43. **Zevenhuizen LPTM, van Neerven ARW.** 1983. (1→2)-β-d-glucan and acidic oligosaccharides produced by *Rhizobium meliloti*. Carbohydrate Research **118:**127-134.
44. **Loewus FA.** 1952. Improvement in anthrone method for determination of carbohydrates. Analytical Chemistry **24:**219.
45. **Meade HM, Long SR, Ruvkun GB.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. J Bacteriol **149:**114-122.
46. **Wells DH, Chen EJ, Fisher RF, Long SR.** 2007. ExoR is genetically coupled to the ExoS-ChvI two-component system and located in the periplasm of *Sinorhizobium meliloti*. Mol Microbiol **64:**647-664.
47. **York GM, Walker GC.** 1997. The *Rhizobium meliloti exoK* gene and *prsD*/*prsE*/*exsH* genes are components of independent degradative pathways which contribute to production of low-molecular-weight succinoglycan. Mol Microbiol **25:**117-134.
48. **Long S, Reed JW, Himawan J, Walker GC.** 1988. Genetic analysis of a cluster of genes required for synthesis of the calcofluor-binding exopolysaccharide of *Rhizobium meliloti*. J Bacteriol **170:**4239-4248.

Table 1. Bacterial strains used in this study

Strain	Relevant characteristic	Source or reference
Sinorhizobium meliloti		
S. meliloti Sm1021	wild-type, SmR derivative of SU47 (Strep^R)	(45)
chvI^{D52E}(EC220)	Succinoglycan gain of function allele in *Sm1021* wild-type background (*chvI*(D52E)/*chvI*$^+$) (Strep^R, Hyg^R)	(25)
chvI^{K214T} (DW570)	Succinoglycan partial loss of function allele (*chvI*(K214T)) (Strep^R, Neo^R)	(46)
exoS96	*Sm1021 exoS96*::Tn5 (Strep^R, Neo^R)	(26)
exoY	*Sm1021 exoY*210::Tn5 (Strep^R, Neo^R)	(47)
exoA	*Sm1021 exoA*32::Tn5-233 (Strep^R, Gent^R)	(7)
*exoS*96/*exoA*	*exoS96-exoA* double mutant (Strep^R, Gent^R, Neo^R)	This study
chvI^{D52E}/*exoY*	*chvI*(D52E)-*exoY* double mutant (Strep^R, Hyg^R)	This study
exoV	*Sm1021 exoV*::Tn5 (*exoV*2), (Strep^R, Neo^R)	(37)
exoH	*Sm1021 exoH::Tn5* (Strep^R, Neo^R)	(34, 48)
exoZ	*Sm1021 exoZ::Tn5* (Strep^R, Neo^R), (Rm8431)	(48)

Figure legends

Figure 1. Hyper activation of *exo* gene regulators protects against NCR247 antimicrobial activity. Early exponential phase cells of the defined strains were treated with 20 μM of NCR247 for five hours in MOPS-GS + cas buffer with NCR247 (A-C). Then the viable cells were enumerated at the definded time-points. Time-points and error bars indicate the mean ± standard deviation. The results shown are representative of trends observed in at least two independent experiments. For space reasons significant differences are only indicated for the five hour time-points. *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$. Data points and error bars indicate the mean ± standard deviation. nt - untreated; ncr - NCR247

Figure 2. Extracellular addition of succinoglycan protects against the antimicrobial activity of cationic NCR247. (A and B) Early exponential phase cells of an *S. meliloti* were treated with 20 μM NCR247 and 10 μg ml^{-1} of the defined succinoglycan preparations for five hours in MOPS-GS + cas buffer and then the number of viable cells was enumerated. Data points and error bars indicate the mean ± standard deviation. Bars and error bars indicate the mean ± standard deviation. The results shown are representative of trends observed in at least two independent experiments. SN – supernatant, HMW – High Molecular Weight, LMW – Low Molecular Weight. *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

Figure 3. *S. meliloti* succinoglycan protects against NCR247 and Bac7. (A) *S. meliloti Sm*1021 were treated with L-NCR247 (20 μM), Polymyxin B (PMB, 1.75 μg ml-1), melittin (1 μM), human defensin 5 (HD5, 20 μM), LL-37 (0.5 μM) or Bac71-35 (0.5 μM) for five hours and the recovered on LBMC agar plates supplemented with 200 μg ml-1 Strep. Results shown are representative of trends observed in at least two independent experiments. (B) Early exponential phase cells of *S. meliloti* wild-type cells were treated with 20 μM of L- or D-NCR247 and supplemented with 10 μg ml^{-1} of HMW wild-type succinoglycan, where indicated. Bars and error bars indicate the mean ± standard deviation. The results shown are

representative of trends observed in at least two independent experiments. White bars – L-NCR247, Black bars – D-NCR247. ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 4. Purified succinoglycan affects NCR247 mobility on a size exclusion column. FI-NCR247 was mixed with 500 µg of the HMW succinoglycan fraction of either the wild-type or *exoV* mutant strains in a final volume of two milliliters and loaded into a BioRad Bio Gel P6 (fine mesh) size exclusion column. The fluorescence of each fraction was measured (488/525 nm) and plotted, not including the void volume. All data is the average of at least three independent experiments.

Figure 5. Graphical representation of the polysaccharide contents of fractions collected from size exclusion chromatography. (A-E) A defined amount of succinoglycan (500 µg) was loaded onto a BioRad BioGel P-6 column and 1.6 ml fractions were collected and the polysaccharide content of each fraction was determined using the anthrone-sulphuric acid assay. 3V – supernatant precipitated with three volumes of ethanol, 7V – 3V fraction precipitated with an additional seven volumes of ethanol.

Figure 6. NCR247 directly interacts with succinoglycan. Octet bio-layer interferometry assays were performed as described in the materials and methods. (A) Overview of all stages of an Octet bio-layer interferometry. These include NCR247-Biotin binding to the biosensor tip and the association of solubilized succinoglycan to immobilized NCR247-biotin. In a final step the laded biosensor tips is immersed in assay buffer without succinoglycan. (B-D) The concentrations of free succinoglycan was kept constant for HMW and LMW forms at 10 µg ml^{-1}. Where indicated, the ammonium acetate assay buffer was supplemented with 0.1 M NaCl. Results shown are represnetative of at least two independent experiments. 1.7-2.7 minutes, buffer; 2.7-8.2 minutes, buffer + HMW or LMW succinoglycan; 8.2-10 minutes, buffer without salt (-NaCl) or with 0.1 M salt (+NaCl) [times vary minimally between experiments]. Results shown are representative of trends observed in at least two independent experiments. EPS – succinoglycan.

Figure 7. HMW succinoglycan from *exoH* and *exoZ* mutants protect against the antimicrobial action of NCR247. Early exponential phase cells of an *S. meliloti* were treated with 20 µM NCR247 and 10 µg ml^{-1} of the defined succinoglycan preparations for five hours in MOPS-GS + cas buffer and then the number of viable cells was enumerated. Data points and error bars indicate the mean ± standard deviation. Bars and error bars indicate the mean ± standard deviation. The results are representative of at least two independent experiments. HMW – High Molecular Weight, *** $p \leq 0.001$, * $p \leq 0.05$. 3V – supernatant precipitated with three volumes of ethanol.

Figure 8. Succinoglycan modifying residues affect polysaccharide binding to NCR247. (A and B) Summary plot of bio-layer interferometry experiments of succinoglycan preparations from *S. meliloti* mutants that are unable to modify succinoglycan with one of the three modifications, succinyl (*exoH*), pyruvyl (*exoV*) or acetyl (*exoZ*). Octet bio-layer interferometry assays were performed as described in the materials and methods. The concentrations of free succinoglycan was kept constant for all derivatives at 10 µg ml^{-1}. Where indicated, the ammonium acetate assay buffer was supplemented with 0.1 M NaCl or the indicated amounts of ethanol. Results shown are representative of trends observed in at least two independent experiments.

Figure 1

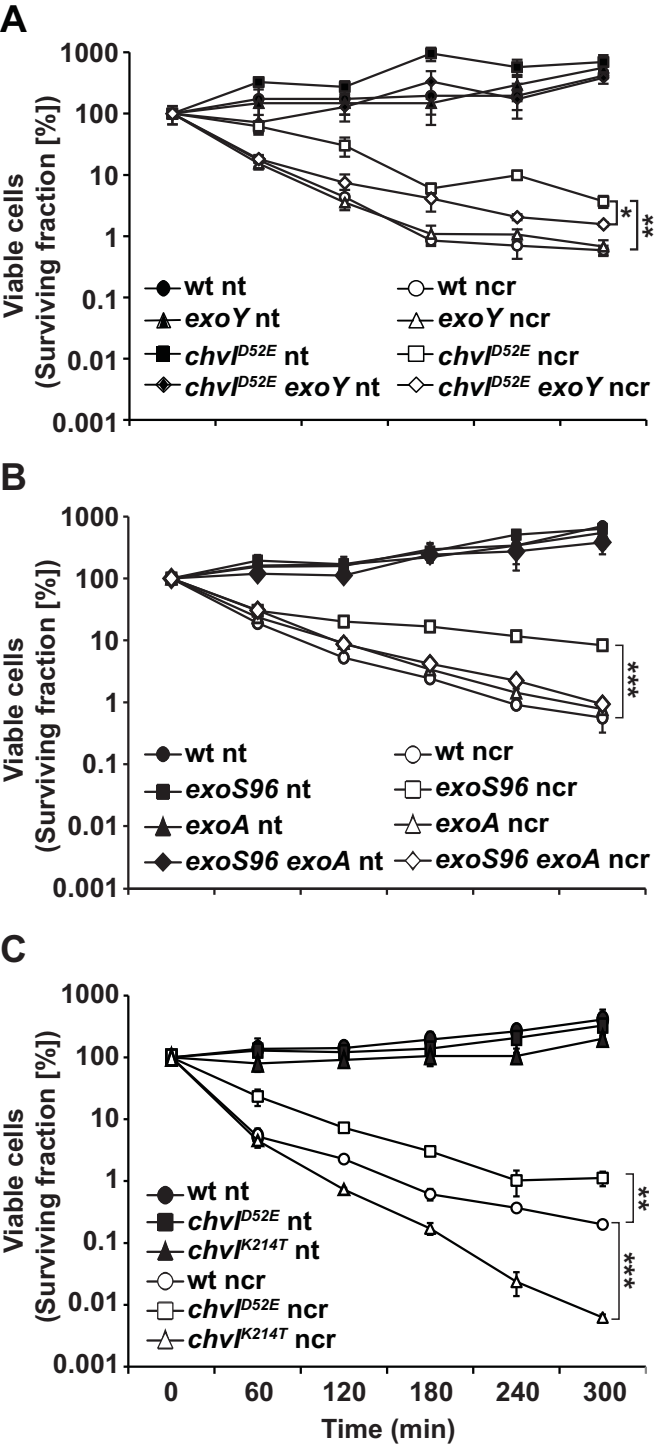

A
Viable cells
(Surviving fraction [%])
1000
100
10
1
0.1
0.01
0.001
wt nt
exoY nt
chvI^D52E nt
chvI^D52E exoY nt
wt ncr
exoY ncr
chvI^D52E ncr
chvI^D52E exoY ncr
*
**
B
Viable cells
(Surviving fraction [%])
1000
100
10
1
0.1
0.01
0.001
wt nt
exoS96 nt
exoA nt
exoS96 exoA nt
wt ncr
exoS96 ncr
exoA ncr
exoS96 exoA ncr
***
C
Viable cells
(Surviving fraction [%])
1000
100
10
1
0.1
0.01
0.001
wt nt
chvI^D52E nt
chvI^K214T nt
wt ncr
chvI^D52E ncr
chvI^K214T ncr
**
***
0
60
120
180
240
300
Time (min)

Figure 2

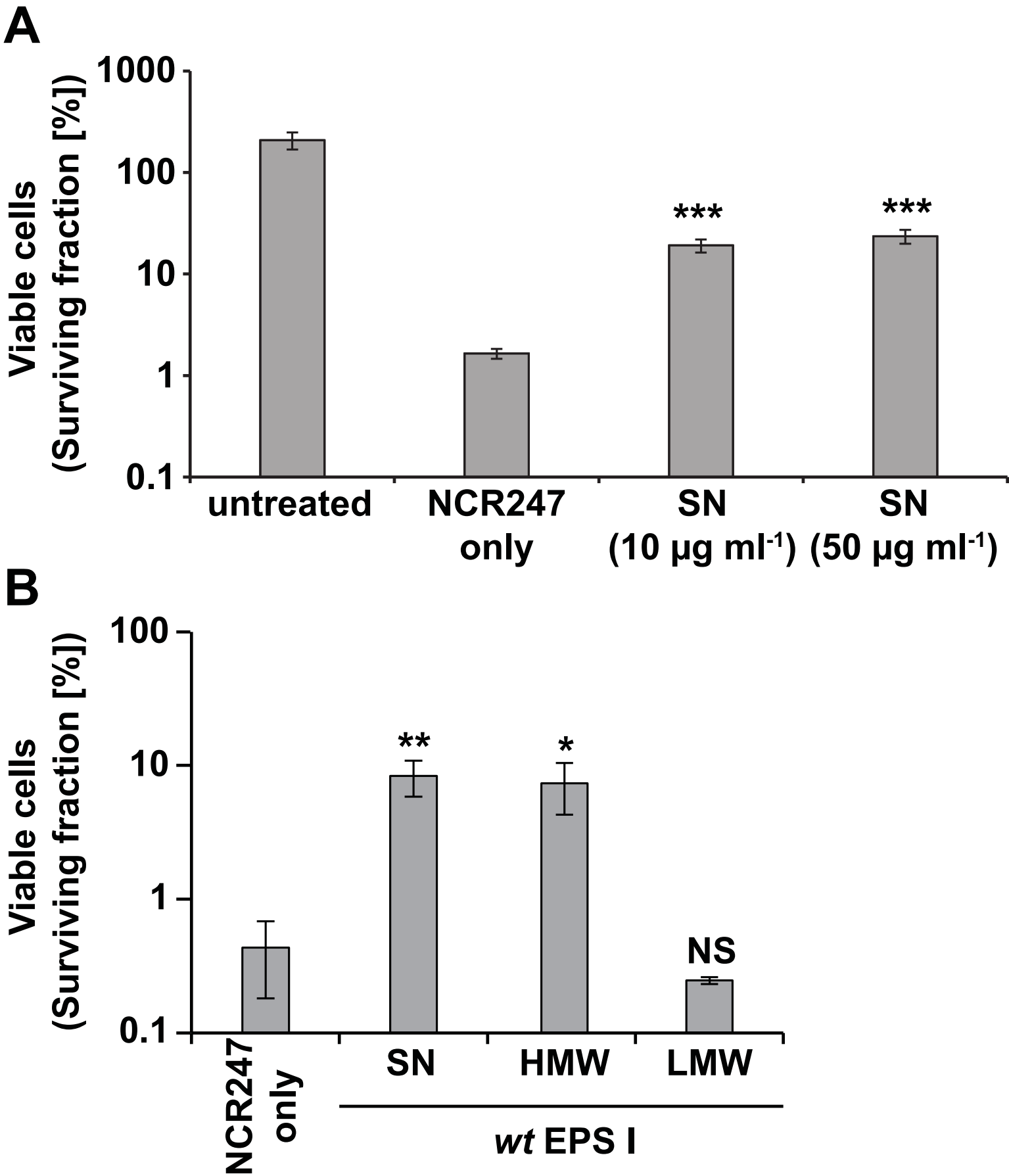

A
Viable cells
(Surviving fraction [%])
1000
100
10
1
0.1
***
***
untreated
NCR247
only
SN
(10 µg ml-1)
SN
(50 µg ml-1)
B
Viable cells
(Surviving fraction [%])
100
10
1
0.1
**
*
NS
NCR247
only
SN
HMW
LMW
wt EPS I

Figure 3

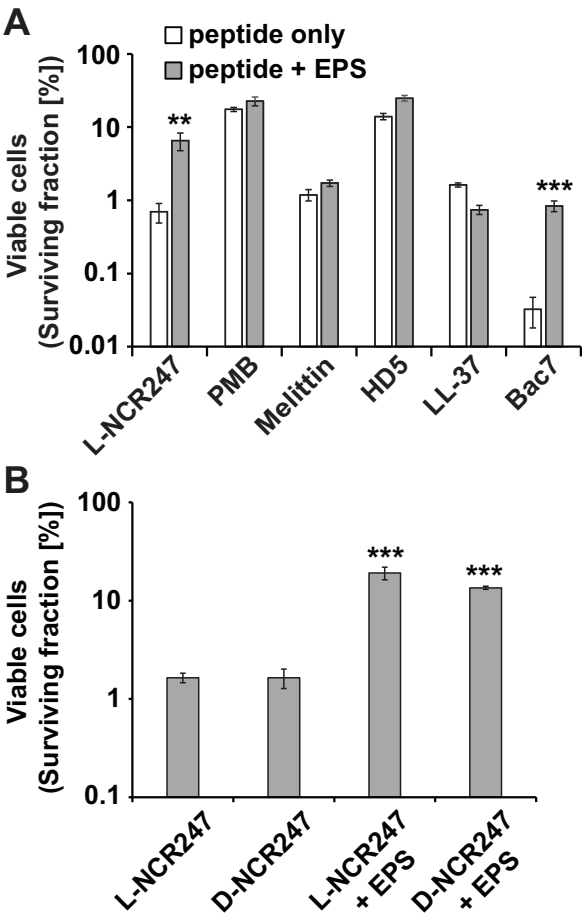

A
peptide only
peptide + EPS
Viable cells
(Surviving fraction [%])
100
10
1
0.1
0.01
**
***
L-NCR247
PMB
Melittin
HD5
LL-37
Bac7
B
Viable cells
(Surviving fraction [%])
100
10
1
0.1
***
***
L-NCR247
D-NCR247
L-NCR247 + EPS
D-NCR247 + EPS

Figure 4

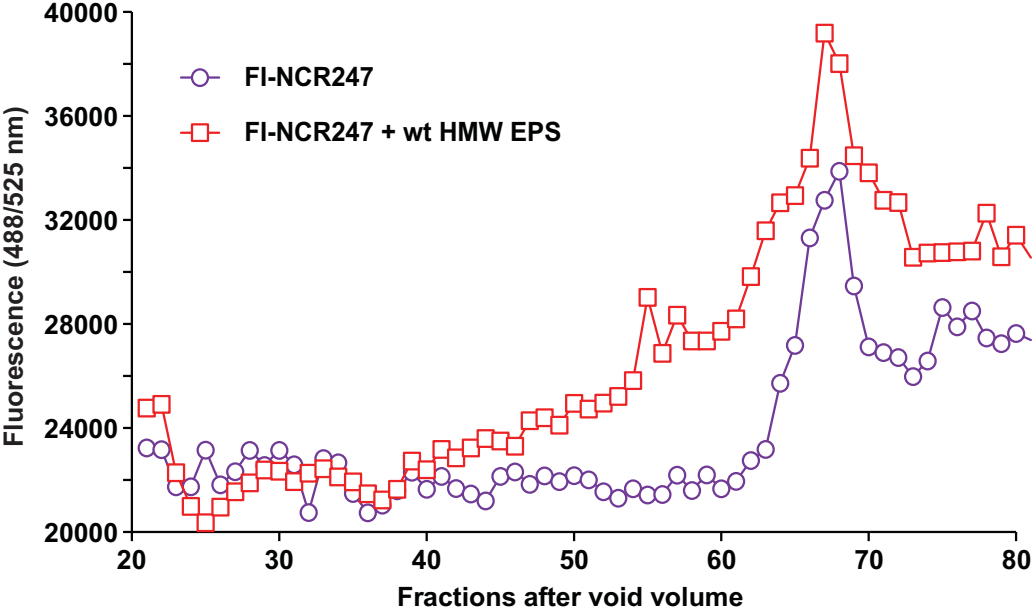

40000
36000
32000
28000
24000
20000
Fluorescence (488/525 nm)
FI-NCR247
FI-NCR247 + wt HMW EPS
20
30
40
50
60
70
80
Fractions after void volume

Figure 5

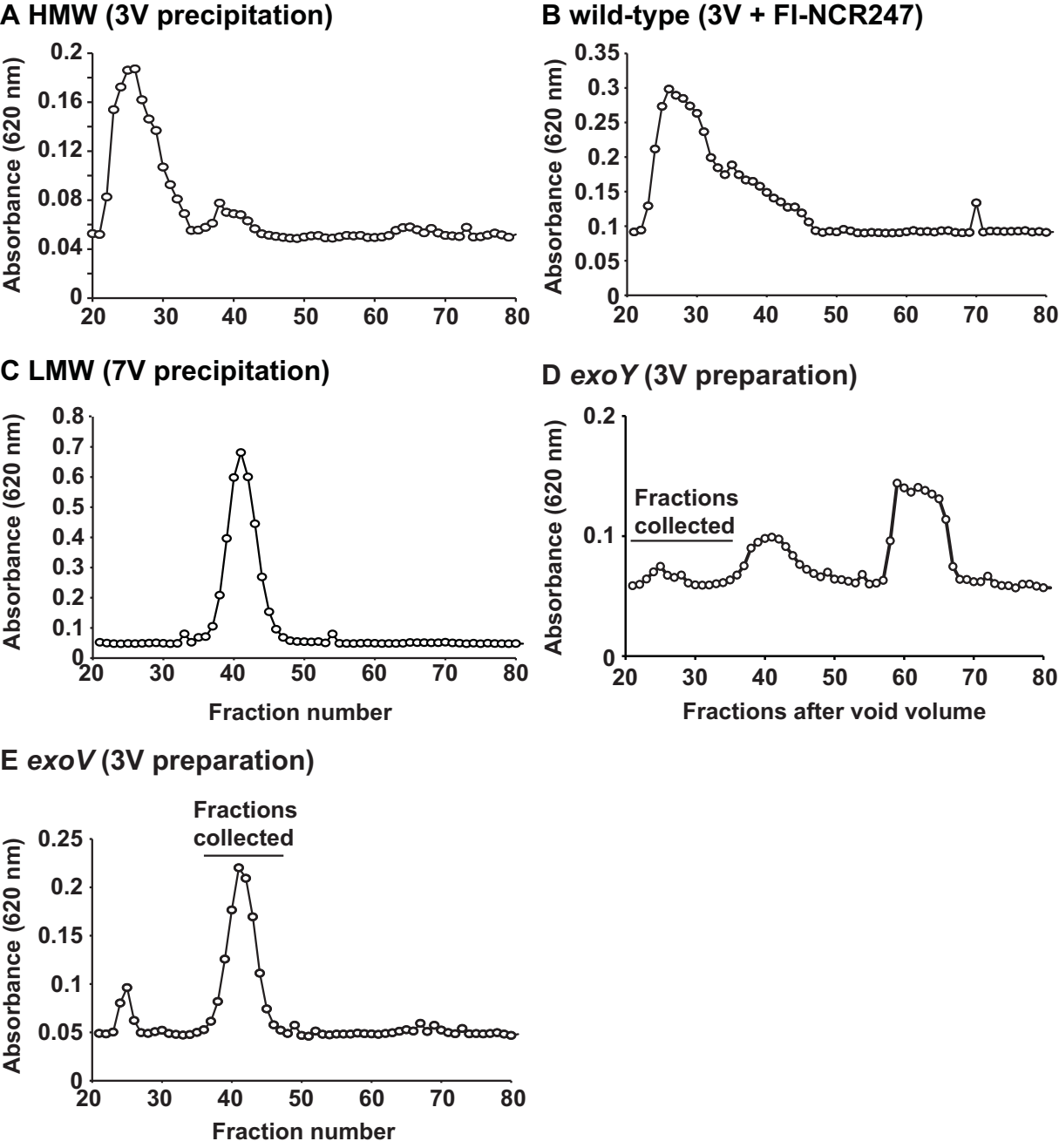

A HMW (3V precipitation)
B wild-type (3V + FI-NCR247)
C LMW (7V precipitation)
D exoY (3V preparation)
E exoV (3V preparation)
Absorbance (620 nm)
Fraction number
Fractions after void volume
Fractions collected

Figure 6

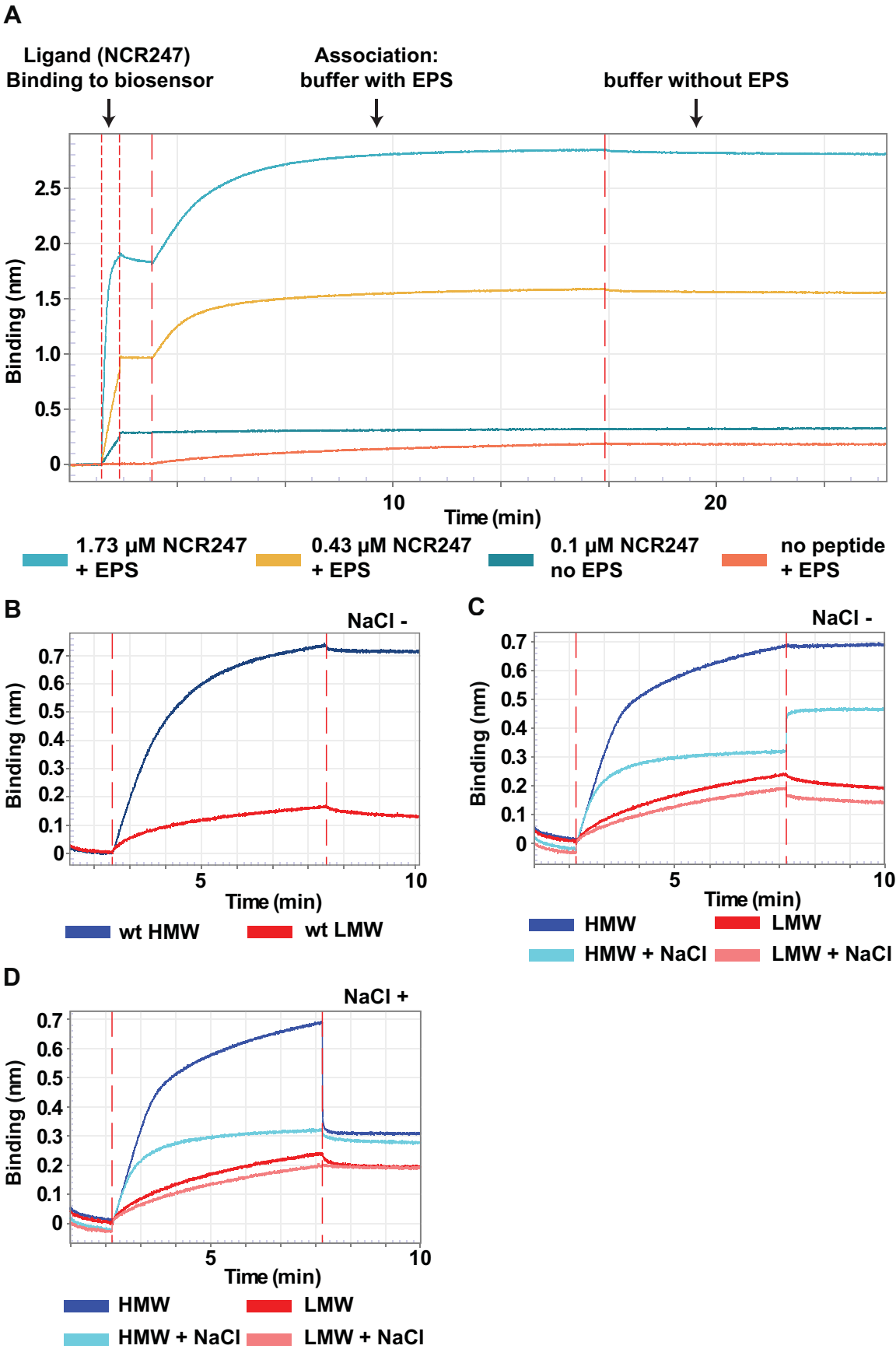

A
Ligand (NCR247)
Binding to biosensor
Association:
buffer with EPS
buffer without EPS
Binding (nm)
Time (min)
1.73 µM NCR247
+ EPS
0.43 µM NCR247
+ EPS
0.1 µM NCR247
no EPS
no peptide
+ EPS
B
NaCl -
Binding (nm)
Time (min)
wt HMW
wt LMW
C
NaCl -
Binding (nm)
Time (min)
HMW
LMW
HMW + NaCl
LMW + NaCl
D
NaCl +
Binding (nm)
Time (min)
HMW
LMW
HMW + NaCl
LMW + NaCl

Figure 7

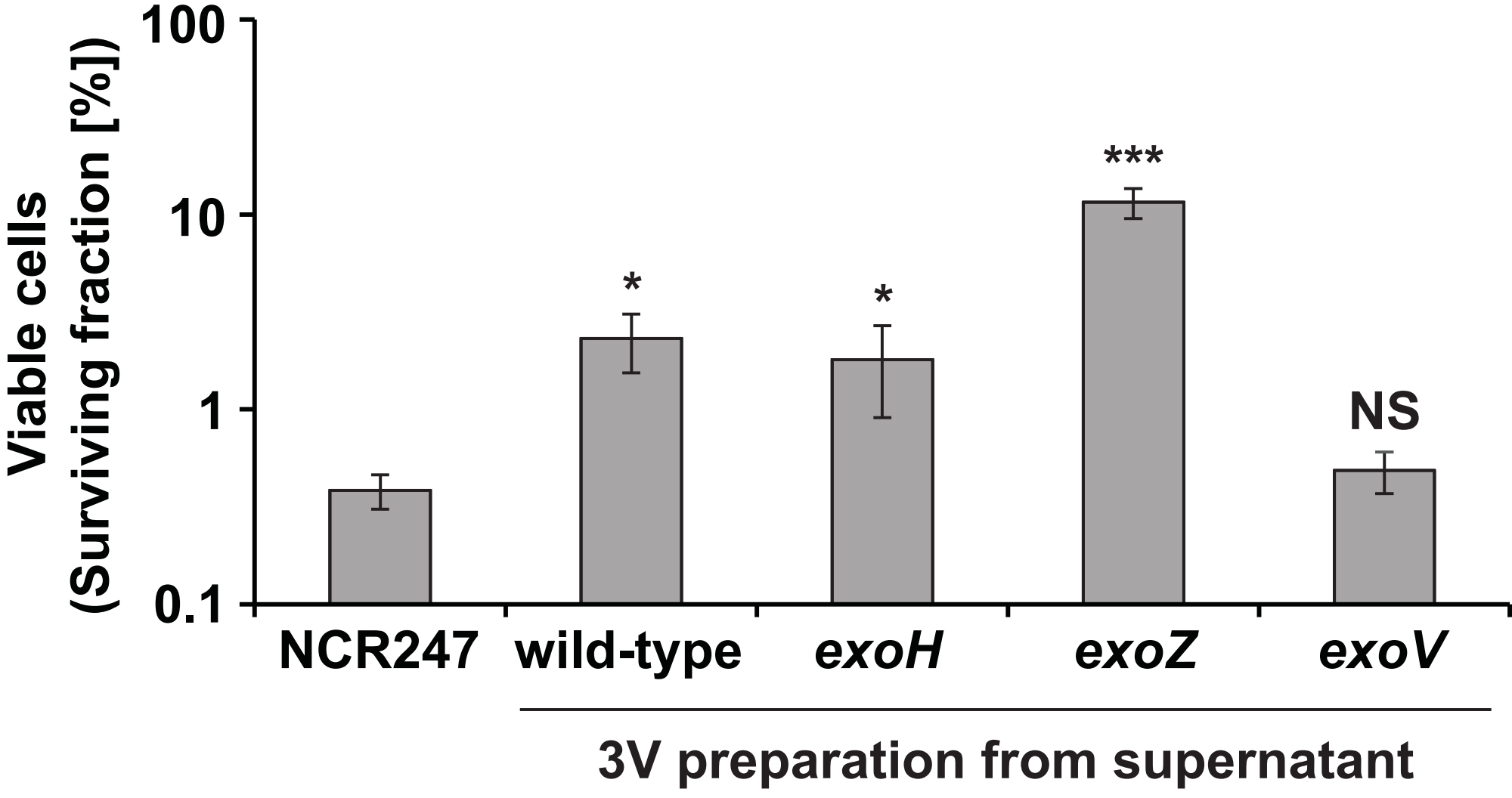
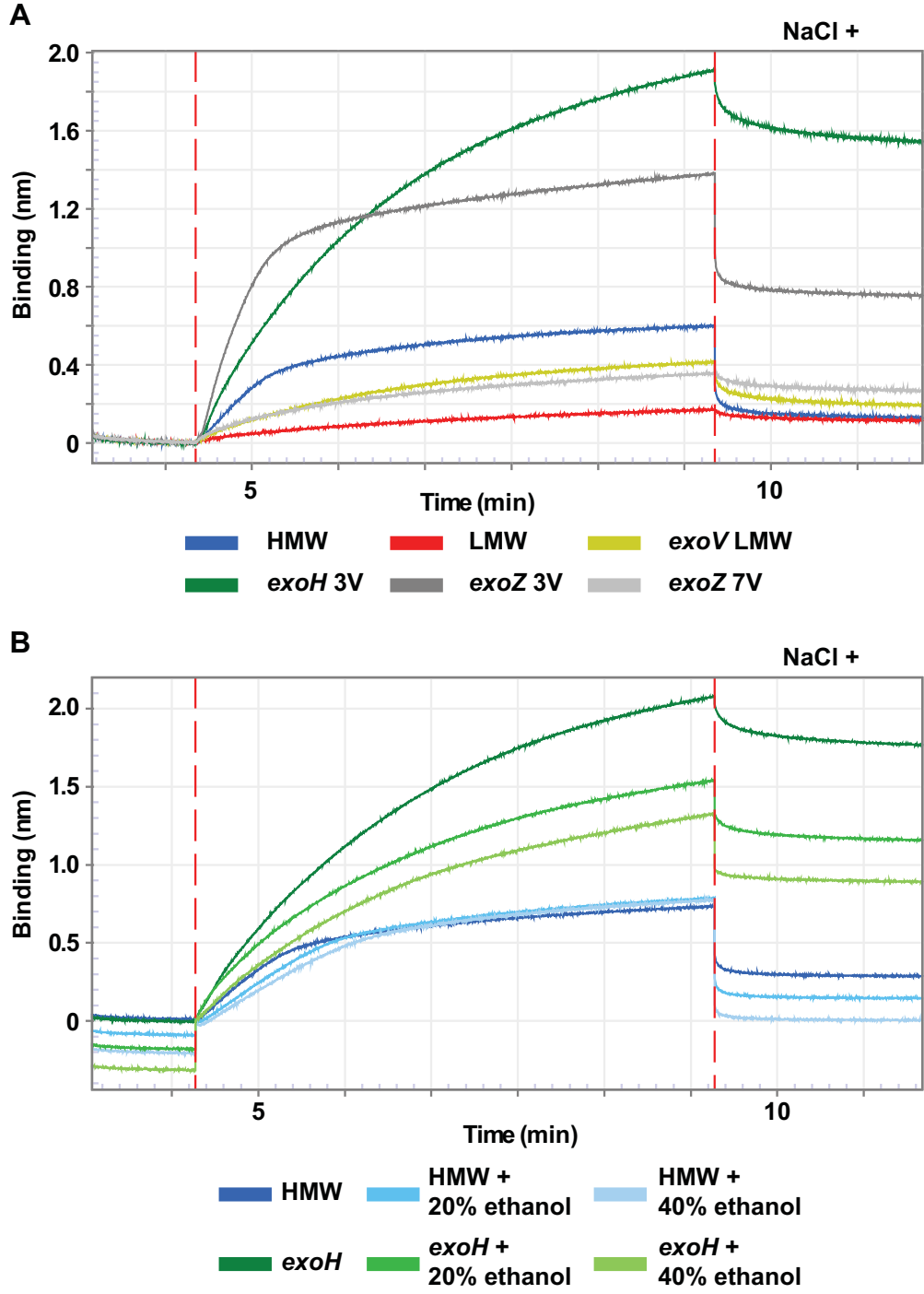

Viable cells
(Surviving fraction [%])
100
10
1
0.1
*
*
***
NS
NCR247
wild-type
exoH
exoZ
exoV
3V preparation from supernatant

Figure 8


A
NaCl +
2.0
1.6
1.2
0.8
0.4
0
Binding (nm)
5
10
Time (min)
HMW
LMW
exoV LMW
exoH 3V
exoZ 3V
exoZ 7V
B
NaCl +
2.0
1.5
1.0
0.5
0
Binding (nm)
5
10
Time (min)
HMW
HMW + 20% ethanol
HMW + 40% ethanol
exoH
exoH + 20% ethanol
exoH + 40% ethanol

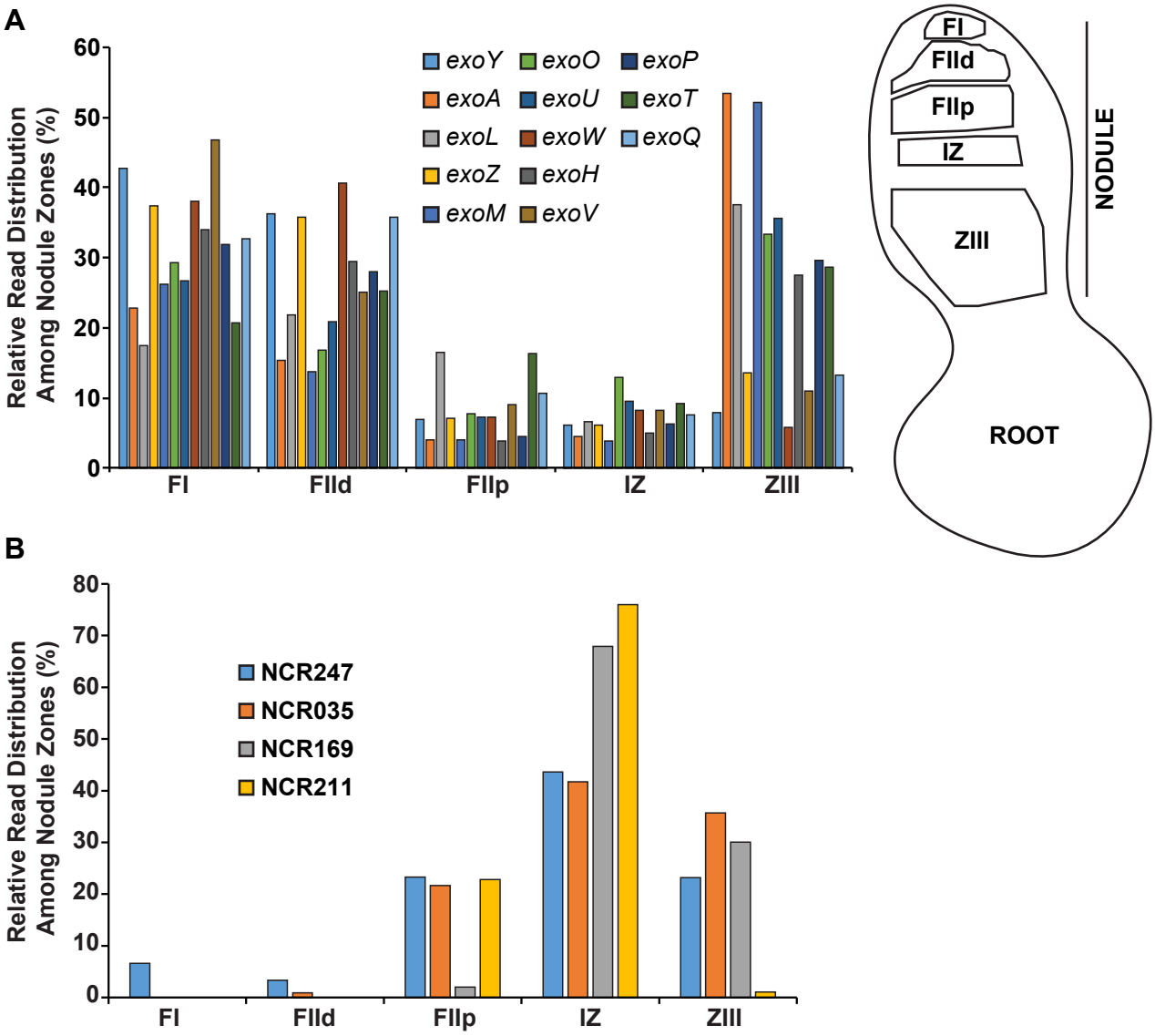


Figure S1. Some *exo* genes show elevated expression levels in the nodule nitrogen fixation zone (ZIII). (A) Relative expression levels of *exo* genes along the symbiotic process represented by distinct nodule sections. (B) Relative expression levels of selected NCR peptide genes along the symbiotic process represented by distinct nodule sections. Data for this figure were derived from Roux *et al.* (2014).

Supplemental Reference

Roux B, Rodde N, Jardinaud MF, Timmers T, Sauviac L, Cottret L, Carrere S, Sallet E, Courcelle E, Moreau S, Debelle F, Capela D, de Carvalho-Niebel F, Gouzy J, Bruand C, Gamas P. 2014. An integrated analysis of plant and bacterial gene expression in symbiotic root nodules using laser capture microdissection coupled to RNA-seq. Plant J 77:817-837.

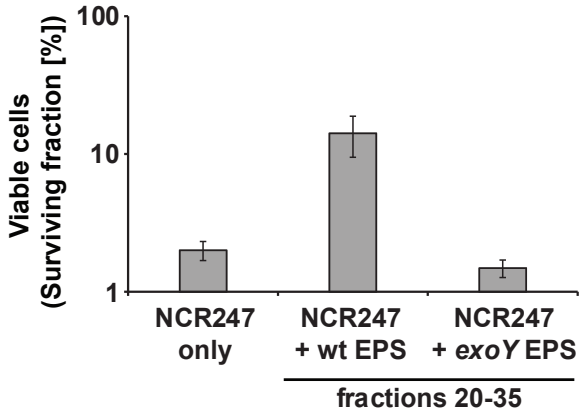


Figure S2. *exoY* mutant supernatant does not contain succinoglycan. Wild-type and *exoY* mutant supernatants were precipitated with three volumes of ethanol and then 500 µg of polysaccharide were separated on a BioGel P-6 size exclusion column. Then the fractions containing HMW succinoglycan (20-35) in wild-type supernatant were collected from both preparations and lyophilized (compare Fig. 5A and D). Lyophilized material from both preparations was then re-suspended in 1 ml of purified H_2O and the polysaccharide concentration of the wild-type fractions determined. *S. meliloti Sm*1021 were treated with 20 µM NCR247 together with either 50 µg ml^{-1} of wild-type peak polysaccharide or the corresponding dilution (same dilution factor as wild-type) of *exoY* mutant fractions 20-35 for 5 hours and viable bacteria were recovered. Bars and error bars indicate the mean ± standard deviation. The results are representative of trends observed in two independent experiments.

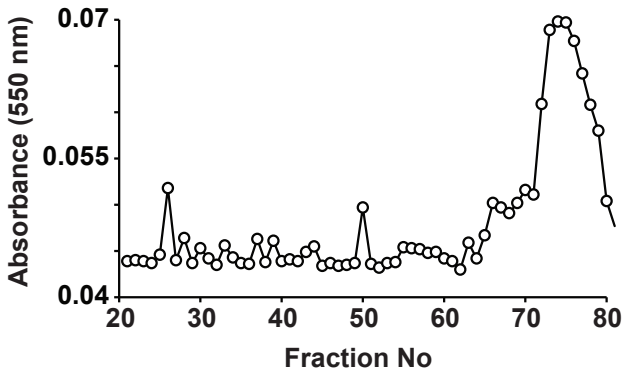


Figure S3. Column volume and polysaccharide distribution after BioGel P-6 column fractionation. 2 ml of 200 µg ml^{-1} $CoCl_2$ in ammonium acetate were loaded onto a BioRad Bio Gel P-6 size exclusion column and 1.6 ml fractions were collected. Then the optical density at 550 nm was determined for every fraction.

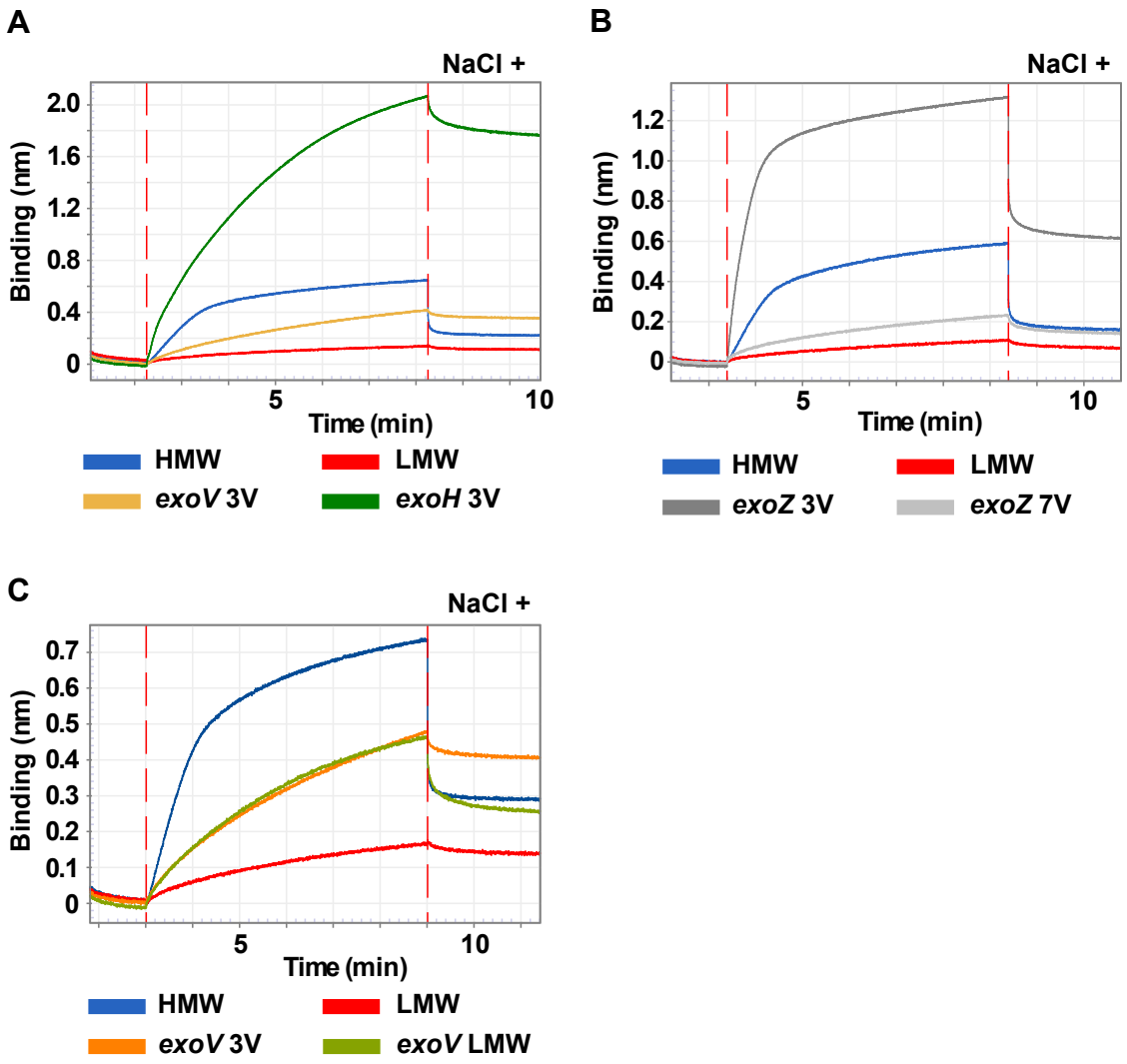


Figure S4. Succinoglycan modificatoins affect molecular binding to NCR247. Octet bio-layer interferometry assays were performed as described in the materials and methods section. The concentrations of free succinoglycan was kept constant for HMW and LMW forms at 10 µg ml^{-1}. Where indicated, the ammonium acetate assay buffer was supplemented with 0.1 M NaCl. Results shown are represnetative of at least two indepenen experiments. HMW - High molecular weight succinoglycan, LMW - Low molecular weight succinoglycan, 3V – supernatant precipitated with three volumes of ethanol, 7V – 3V fraction precipitated with an additional seven volumes of ethanol.