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Inhibiting sulfate-reducing bacteria in biofilms by expressing the antimicrobial peptides indolicidin and bactenecin

A Jayaraman[1], FB Mansfeld[2] and TK Wood[3]

[1]*Center for Engineering in Medicine, Massachusetts General Hospital, and Harvard Medical School, Boston, MA 02114;* [2]*Materials Science & Engineering, University of Southern California, Los Angeles, CA 90089;* [3]*Department of Chemical Engineering, University of Connecticut, Storrs, CT 06269, USA*

To identify novel, less-toxic compounds capable of inhibiting sulfate-reducing bacteria (SRB), *Desulfovibrio vulgaris* and *Desulfovibrio gigas* in suspension cultures were exposed to several antimicrobial peptides. The bacterial peptide antimicrobials gramicidin S, gramicidin D, and polymyxin B as well as the cationic peptides indolicidin and bactenecin from bovine neutrophils decreased the viability of both SRB by 90% after a 1-h exposure at concentrations of 25–100 μg ml^{-1}. To reduce corrosion by inhibiting SRB in biofilms, the genes for indolicidin and bactenecin were expressed in *Bacillus subtilis* BE1500 and *B. subtilis* WB600 under the control of the constitutive alkaline protease (*apr*) promoter, and the antimicrobials were secreted into the culture medium using the *apr* signal sequence. Bactenecin was also synthesized and expressed as a fusion to the pro-region of barnase from *Bacillus amyloliquefaciens*. Concentrated culture supernatants of *B. subtilis* BE1500 expressing bactenecin at 3 μg ml^{-1} decreased the viability of *Escherichia coli* BK6 by 90% and the reference SRB *D. vulgaris* by 83% in suspension cultures. *B. subtilis* BE1500 and *B. subtilis* WB600 expressing bactenecin in biofilms also inhibited the SRB-induced corrosion of 304 stainless steel six to 12-fold in continuous reactors as evidenced by the lack of change in the impedance spectra (resistance polarization) upon addition of SRB and by the reduction in hydrogen sulfide and iron sulfide in batch fermentations with mild steel. A 36-fold decrease in the population of *D. vulgaris* in a *B. subtilis* BE1500 biofilm expressing bactenecin was also observed. This is the first report of an antimicrobial produced in a biofilm for *in vivo* applications and represents the first application of a beneficial, genetically-engineered biofilm for combating corrosion.

Keywords: engineered biofilms; biocorrosion; sulfate-reducing bacteria

Introduction

Sulfate-reducing bacteria have been widely implicated in the anaerobic corrosion of steel and copper alloys [15,18,38] (US losses at $4–6 billion/year [5]). Conventional methods of biofilm control and removal in an industrial setting are generally inadequate against the adherent biofilm bacteria [8,17]. The exopolysaccharide matrix secreted by the cells is considered to be involved in reducing the reactivity of biocides by preventing them from penetrating the biofilm [7]. Attempts to circumvent this problem have focused on using very high concentrations of biocides which may in turn pose an environmental hazard [9]. Therefore, it may prove advantageous to generate antimicrobials at the biofilm-substrate interface. P Wood *et al* [39] recently reported that generation of hydrogen peroxide and potassium monopersulfate at the colonized surface increased the sensitivity of a *Pseudomonas aeruginosa* biofilm by 150-fold. Jansen and Kohnen [19] suggest that modifying polymer surfaces and impregnating them with silver ions may reduce *Staphylococcus epidermis* KH6 attachment. Bayston *et al* [4] reported that impregnating catheters with rifampicin and clindamycin inhibited colonization and protected them against three successive additions of *S. epidermis* over a 28-day period. These studies suggest generating antimicrobials from within the biofilm might exclude SRB in industrial settings. Since some peptide antimicrobials are relatively small (which aids in protein engineering [30]), they were chosen for expression in biofilm-forming aerobic bacteria for excluding SRB from biofilms.

Saleh [34] and Postgate [31] compiled lists of antimicrobials which are inhibitory to various SRB which include the peptide polymyxin B (inhibits *Desulfovibrio vulgaris* at 100 μg ml^{-1}). More recently, antimicrobial peptides have been identified and isolated from several bacteria, plants, insects, amphibians, and mammals and have antimicrobial activity against Gram-negative and Gram-positive bacteria as well as fungi [16].

Indolicidin [12,35] and bactenecin [13,33] are cationic antimicrobial peptides isolated from bovine neutrophils. Indolicidin is a tridecapeptide which belongs to the family of defensins and consists only of six different amino acids with the highest proportion of tryptophan (39%) in any known protein [12]. Indolicidin is also the smallest known linear antimicrobial peptide, and its carboxyl terminus is amidated in its naturally occurring form. Bactenecin is an arginine-rich, cyclic dodecapeptide and contains a disulfide bond which maintains the cyclic structure [33].

Few attempts have been made at producing antimicrobial peptides in various expression systems for commercial applications [29,30]. These approaches target large-scale, inexpensive production of purified antimicrobial peptides rather than *in vivo* applications.

Correspondence: Dr TK Wood, Department of Chemical Engineering, University of Connecticut, Storrs, CT 06269, USA
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The present study describes inhibition of the representative SRB *D. vulgaris* and *D. gigas* in suspension cultures by the peptide antimicrobials gramicidin S (10-amino acid cyclic peptide from *Bacillus brevis* [3]), gramicidin D (15-amino acid linear peptide from *B. brevis* [37]), amidated and non-amidated indolicidin, bactenecin, and polymyxin B (10-amino acid branched cyclic decapeptide from *B. polymyxa* [14]). This is the first report of the inhibition of SRB with the cationic antimicrobial peptides indolicidin, bactenecin, and gramicidin S. To exclude SRB in biofilms on 304 stainless steel to reduce corrosion, indolicidin and bactenecin have been cloned as fusions to the alkaline protease (*apr*) signal sequence and expressed constitutively using the *apr* promoter in *Bacillus* strains. The pro-region of barnase (an extracellular RNase from *B. amyloliquefaciens*) has also been utilized to produce bactenecin as a pre-pro-peptide. The ability of these genetically engineered *Bacillus* strains to inhibit the growth of SRB by secreting antimicrobials was characterized using concentrated supernatant phases of the recombinant strains to inhibit exponential growth of SRB in suspension cultures and by measuring the reduction of SRB populations in biofilms consisting of the representative SRB *D. vulgaris* and the protective recombinant strains. The practical application of the principle of protective bacteria was demonstrated by reducing SRB-related corrosion of 304 stainless steel in continuous fermentations (200–300 h) using electrochemical impedance spectroscopy.

Materials and methods

Bacterial strains, plasmids, and growth media

Escherichia coli XLI (Blue) {*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F′ proAB lacI*^{q}*ZΔM15* Tn*10(Tet*^{r})]} was purchased from Stratagene, La Jolla, CA, USA. *B. subtilis* BE1500 {*trpC2, metB10, lys-3, Δ aprE66, Δ npr-82, Δ sacB::ermC*} and plasmid pBE92 containing the alkaline protease (*apr*) promoter, signal sequence, and the alkaline phosphatase reporter gene were obtained from EI du Pont de Nemours Inc, Wilmington, DE, USA. The protease-deficient strain *B. subtilis* WB600 [40] {*trpC2, Δ nprE, Δ aprA, Δ epr, Δ bpf, Δ mpr, Δ nprB*} was obtained from Dr Sui-Lam Wong, University of Calgary, Alberta, Canada. *D. vulgaris* (ATCC 29579) and *D. gigas* (ATCC 19364) were obtained from the American Type Culture Collection and cultivated in 15-ml screwcap tubes with 10 ml of modified Baars' medium (ATCC medium 1249) supplemented with 100 μl each of the oxygen-scavengers, 4% sodium sulfide and Oxyrase (Oxyrase Inc, Mansfield, OH, USA). Initial cultures were grown from –85°C glycerol stocks; all subsequent cultures were grown with a 3% inoculum from the initial culture maintained at 30°C without shaking. Both SRB were routinely cultured in tightly-closed screwcap tubes after exposure to oxygen during cultivation (which did not inhibit cultivation and agrees with the results of Angell and White [1]). SRB were also cultured periodically in the presence of 0.1% ferrous ammonium sulfate, and the presence of these sulfate-reducers was confirmed by the detection of black iron sulfide in the culture tubes. The desulfoviridin assay was also performed after each MPN assay to confirm the presence of *D. vulgaris* or *D. gigas* by its red color under UV light due to the release of the chromophore of the pigment desulfoviridin [31]. All corrosion experiments with engineered *B. subtilis* strains and *Pseudomonas fragi* K [21] were carried out in modified Baars' medium (ATCC medium 1249) to facilitate growth of the sulfate-reducing bacteria.

Antimicrobial peptides and chemicals

Indolicidin (amidated and free-acid form) was kindly provided by Professor Michael E Selsted of the University of California, Irvine, and additional free-acid form was synthesized by Genosys Biotechnologies Inc, The Woodlands, TX, USA at 76% purity. Gramicidin S (96.5% purity), gramicidin D (100% purity), and polymyxin B (100% purity) were purchased from Sigma Chemical Co, St Louis, MO, USA. Bactenecin was synthesized by Genosys Biotechnologies Inc at 32% purity, and shipped in the presence of dithiothreitol (DTT, $<$0.1%). The molecular weights of the synthesized indolicidin (acid form, 1907 Da) and bactenecin (1486 Da) were verified using a MALDI-Time of Flight (TOF) mass spectrometer (Voyager DE 5–2386–00, Perseptive Biosystems, MA, USA). A Vydac C18 column (Vydac, Hesperia, CA, USA) was used on a reverse-phase HPLC (Varian Vista 5000 series, Sugar Land, TX, USA) to remove the residual DTT from bactenecin and to facilitate the formation of a disulfide bond between residues 3 and 11. Enzymes were obtained from Promega, Madison, WI, USA. BCIP (5-bromo-4-chloro-3-indolyl phosphate) was purchased from Sigma Chemical Co.

Antimicrobial assays

For determining the viability indices [33] of SRB, a late-exponential phase culture (OD_{600} 0.16–0.19 which corresponded to an initial cell number of $5–9 \times 10^4$ cells ml^{-1}) was exposed anaerobically to various concentrations of antimicrobials for 1 h at 30°C. One milliliter of cells was harvested, washed once in fresh modified Baars' medium to remove cellular debris, and resuspended in 1 ml of fresh modified Baars' medium supplemented with 10 μl each of Oxyrase and 4% sodium sulfide. Aliquots of 450 μl were dispensed in 500-μl sterile microcentrifuge tubes, appropriate amounts of antimicrobials added and the suspension was incubated at 30°C. The effectiveness of treatment was determined by the multiple-tube most-probable number (MPN) fermentation technique and Thomas formula [2] using three 12-ml tubes with a 1000-μl inoculum of SRB, three 12-ml tubes with a 500-μl inoculum, and three 12-ml tubes with a 100-μl inoculum. All nine tubes contained a final volume of 10 ml of modified Baars' medium supplemented with 100 μl each of 4% sodium sulfide and Oxyrase. The tubes were monitored for 72 h to determine the number of tubes that were positive for growth.

To determine the susceptibility of the *B. subtilis* hosts to the expressed antimicrobials, these strains were grown from a single colony in 25 ml of Luria Bertani [24] (LB) medium with shaking at 37°C to an OD_{600} of 0.40–0.45. One-milliliter aliquots were collected, washed with fresh LB medium and resuspended in 100 μl of fresh LB medium in sterile Eppendorf tubes. The antimicrobials indolicidin (in non-amidated form, Ind-OH) and bactenecin were added (50–100 μg ml^{-1}) and the tubes were incubated at 30°C for 1 h

without shaking. Appropriate dilutions were spread on LB agar plates and incubated overnight at 37°C to determine the extent of survival. The results were confirmed by two independent experiments.

Expression of indolicidin and bactenecin in *Bacillus* was determined in duplicate by exposing *E. coli* BK6 in suspension to 25-fold concentrated culture supernatants. *E. coli* BK6 was grown to an OD_{600} of 0.20–0.25, pelleted at room temperature, and resuspended in different volumes (50 or 100 μl) of the concentrated supernatant. The cell suspension was incubated at 30°C for 1 h without aeration, and appropriate dilutions were plated on LB agar plates to determine the antimicrobial activity of the supernatant.

The ability of the supernatant from the *B. subtilis* constructs to inhibit SRB in suspension was determined by resuspending 500 μl of a late-exponential phase *D. vulgaris* culture (OD_{600} = 0.15–0.20) in an equal volume of 25-fold concentrated culture supernatant from *B. subtilis* BE1500, with the antimicrobial plasmids under anaerobic conditions, by adding 10 μl each of Oxyrase and 4% sodium sulfide. The cells were incubated at 30°C for 1 h, and the surviving SRB enumerated using the three-tube MPN assay [2].

To determine the extent of the inhibition of SRB in a biofilm of recombinant *Bacillus* expressing antimicrobials, a biofilm was formed with 2 days growth of *B. subtilis* BE1500 with the appropriate antimicrobial plasmids on 304 stainless steel coupons (2.5 cm diameter, 1.2 mm thickness) in Baars' medium. A 1% inoculum of *D. vulgaris* was added and allowed to grow for 5 days. To find the number of viable SRB in the biofilm by the three-tube MPN method, the biofilm was rinsed once in sterile water to remove loosely attached cells, scraped from the 304 stainless steel coupons, resuspended, and serially diluted in fresh modified Baars' medium under anaerobic conditions (using 10 μl each of Oxyrase and 4% sodium sulfide). The number of aerobic bacteria in the biofilm was determined by plating appropriate dilutions on LB agar plates.

Plasmid construction

Recombinant DNA methods were performed as described by Maniatis [24] and Rodriguez and Tait [32]. Plasmid DNA was isolated from *Bacillus* strains according to the procedure of Bramucci and Nagarajan [6]. The amino acid sequences for non-amidated indolicidin (NH_2-Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-OH) [35] and bactenecin (NH_2-Arg-Leu-Cys-Arg-Ile-Val-Val-Ile-Arg-Val-Cys-Arg-OH) [33] were used to design oligonucleotides which encode the genes for these peptides. Plasmid pBE92-Ind was designed to express non-amidated indolicidin as a 13-amino acid peptide fused to the *apr* signal sequence; pBE92-Bac was designed to express bactenecin as a 12-amino acid peptide fused to the *apr* signal sequence; and pBE92-ProBac was designed to express bactenecin fused to the proregion of the extracellular RNase barnase from *B. amyloliquefaciens* [28] and the *apr* signal sequence.

The synthetic oligos (Figure 1a) were synthesized by Gibco-BRL Life Technologies, Long Island, NY, USA, with flanking *Hin*d III and *Nhe* I restriction sites with an additional six bases at either end for efficient restriction digestion. Two fully complementary oligos of each construct were annealed and digested with *Hin*d III and *Nhe* I. Plasmid vector pBE92 was isolated from *E. coli* XLI (Blue) cell extracts, digested with *Hin*d III, *Nhe* I, and *Sal* I simultaneously, and ligated to the restricted, annealed oligos.

Probactenecin was synthesized as two oligo strands with a 21-base pair complementary region with *Hin*d III and *Nhe* I restriction sites at the ends of the two strands (Figure 1b). A *Not* I site was also engineered downstream of the stop codon which served to introduce an unique site into pBE92-ProBac. The two strands were annealed, and the complementary regions completed using *Taq* polymerase (one cycle, 30 s at 94°C, followed by 30 s at 55°C, and 2 h at 72°C) with a Perkin-Elmer thermal cycler N801-0150, Perkin Elmer, Norwalk, CT, USA.

Transformants containing the correct insert (pBE92-Ind, pBE92-Bac, and pBE92-ProBac) were screened as white colonies on LB agar plates containing 100 μg ml^{-1} of ampicillin and 40 μg ml^{-1} of BCIP (transformants with the correct insert produced white colonies while the reclosed vector resulted in blue colonies). The plasmids containing inserts were further characterized through restriction digests with *Bgl* I (indolicidin), *Bss*H II (bactenecin), and *Not* I (probactenecin), and confirmed using a modification of the Boehringer-Mannheim colony-lift assay in which the inserted genes were detected using antimicrobial gene synthetic oligo DNA (70 bp, Figure 1).

Transformation of E. coli and Bacillus

E. coli XLI (Blue) cells were made electrocompetent according to the method of Smith and Iglewski [36] and electroporated using a gene pulser/pulse controller (Bio-Rad Laboratories, Hercules, CA, USA). *B. subtilis* strains were transformed according to the two-step method of Cutting and Vander Horn [11] and plated on LB agar plates containing kanamycin (25 μg ml^{-1} for BE1500, 50 μg ml^{-1} for WB600).

Batch and continuous corrosion experiments

Batch culture corrosion experiments using SAE 1018 mild steel coupons (2.5 cm diameter, 1.2 mm thick) were performed in triplicate in 250-ml Erlenmeyer flasks at 30°C without shaking as described previously [21]. Seventeen 200–300 h continuous reactor experiments [20] were also used to quantify *in situ* expression of the antimicrobials; biofilms were developed on 304 stainless steel and corrosion was monitored using electrochemical impedance spectroscopy (EIS) with a Solarton-Schlumberger electrochemical measurement unit (SI 1280, Schlumberger Technical Instruments Division, San Jose, CA, USA) interfaced to a PowerMac 7100/80 (Apple Computers, Cupertino, CA, USA). The open circuit potential was measured as the potential between the metal specimen and an Ag/AgCl reference electrode, and the polarization resistance (R_p) was determined as the low frequency value of the impedance (where the imaginary part of the impedance was zero or negligible). Continuous culture corrosion rates were estimated as the inverse of the polarization resistance [20]. A 1% inoculum of a 15 to 18-h SRB culture was added to the reactors after 3–5 days of aerobic *Bacillus* biofilm development.

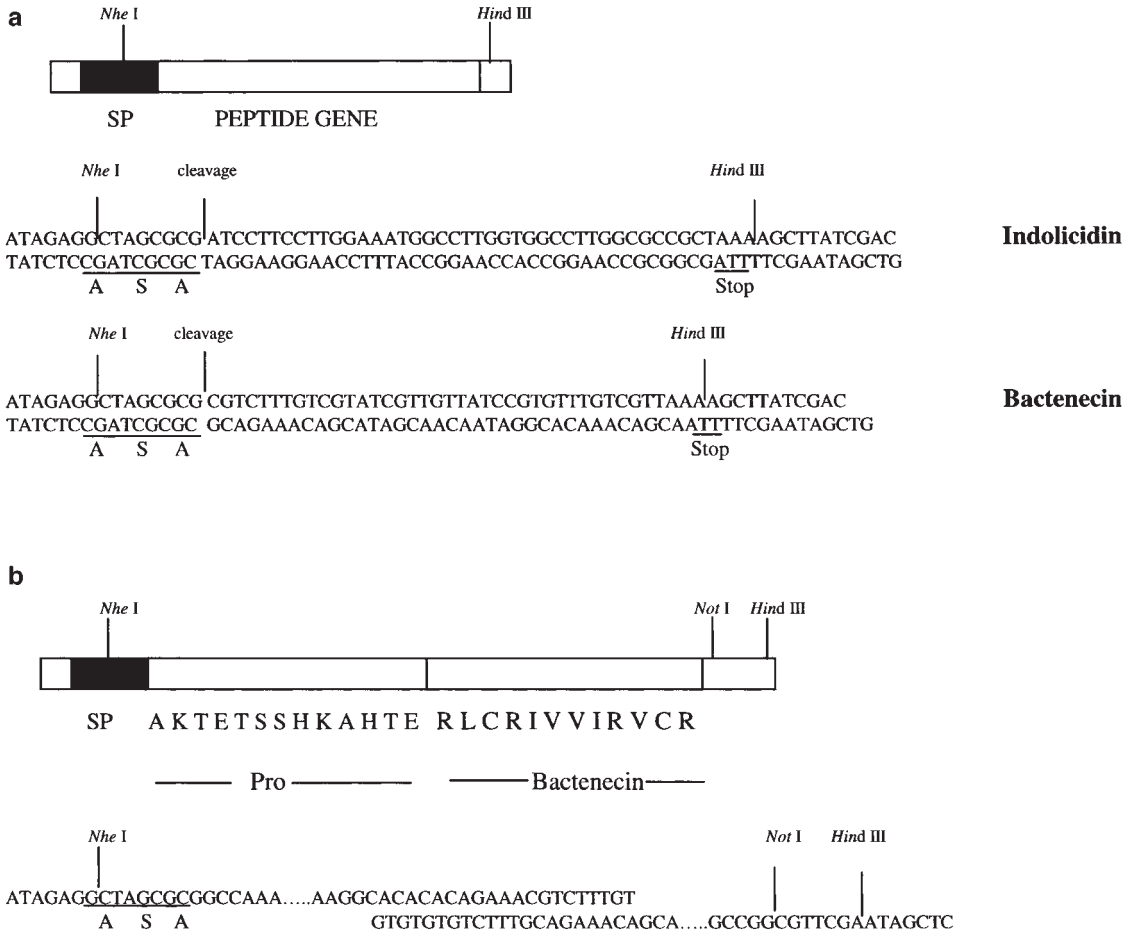

Figure 1 Schematic of expression system and complementary oligonucleotides used for cloning and secreting indolicidin and bactenecin (a) and bactenecin with a protective pro-barnase (pro) region (b). One-letter amino acid codes in (b) represent the pro-region and the bactenecin gene. SP denotes the alkaline protease signal peptide, S = Serine and A = Alanine. Only relevant restriction sites are shown.

Results

Identifying SRB antimicrobial peptides

D. vulgaris and *D. gigas* were incubated in the presence of various antimicrobial peptides, and their viability after a 1-h exposure was determined. Ampicillin was used as a positive control for *D. vulgaris* as it and chloramphenicol inhibited this strain at 20 μg ml^{-1} which agreed with previous reports [27,31]; however, neither ampicillin nor chloramphenicol were effective in inhibiting *D. gigas* at 100 μg ml^{-1}. The susceptibility of both SRB to several additional antibiotics (kanamycin, tetracycline, thiostrepton, penicillin G, and naladixic acid), inorganics (ammonium molybdate, sodium molybdate, and anthraquinone), and peptides (nisin and polymyxin B) was also evaluated using stationary-phase cultures of SRB; *D. gigas* was inhibited by anthraquinone at 100 μg ml^{-1} (as reported earlier [10], and both SRB were inhibited by sodium molybdate at 100 μg ml^{-1}. This is similar to the observation of Saleh [34], who surveyed nearly 200 compounds for their SRB-inhibitory activity and noted that they show a high degree of resistance to inhibitory compounds [31,34].

The MPN assay was used to determine the viability index of *D. gigas* and *D. vulgaris* for the peptide antimicrobials (Figures 2 and 3). For *D. gigas*, both gramicidin S and the amidated form of indolicidin, Ind-NH$_2$ (which is the naturally occurring form in bovine neutrophils [12,35]), reduced the viability of a late-exponential-phase culture by 92–96% after a 1-h exposure at 25 μg ml^{-1} (Figure 2). For *D. vulgaris*, Ind-NH$_2$ at 25 μg ml^{-1} was slightly more effective in inhibiting growth (viability reduced by 99.3%), while gramicidin S was less effective and reduced viability by 93% at 100 μg ml^{-1} (Figure 3). The acid form of indolicidin (Ind-OH) was 10-fold less effective than the amidated form of indolicidin against *D. gigas* and 174-fold less effective against *D. vulgaris* at 25 μg ml^{-1}. This is not surprising as the post-translational amidation is thought to increase the potency of indolicidin [12]. The peptide antimicrobials gramicidin D, polymyxin B, and bactenecin also decreased the viability of *D. vulgaris* and *D. gigas* by approximately 90% at 100 μg ml^{-1}. These MPN assay results were also corroborated by the similar results obtained (data not shown) when *D. vulgaris* was exposed to gramicidin S, gramicidin D, indolicidin, and bactenecin

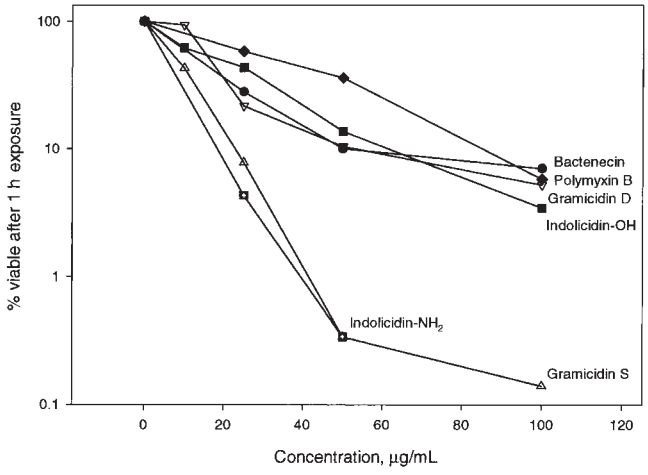

Figure 2 Viability curves of *D. gigas* upon exposure of 450 μl of late-exponential phase culture (OD_{600} = 0.16–0.19) to antimicrobial peptides indolicidin-OH, indolicidin-NH_2, bactenecin, polymyxin B, gramicidin D, and gramicidin S for 1 h at 30°C. Cell numbers were determined by the MPN method.

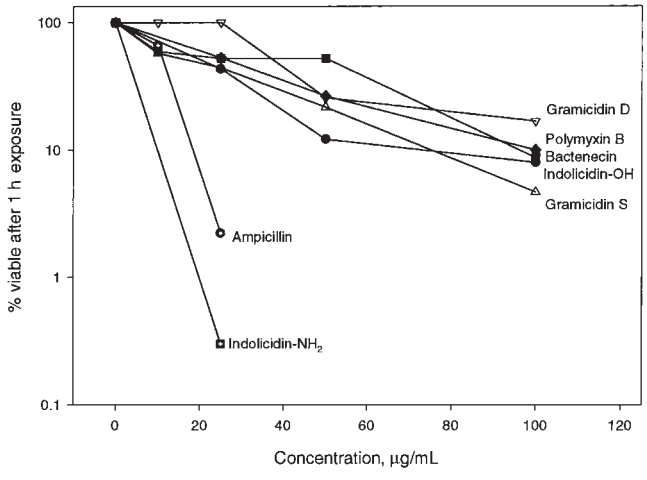

Figure 3 Viability curves of *D. vulgaris* upon exposure of 450 μl of late-exponential phase culture (OD_{600} = 0.16–0.19) to ampicillin and antimicrobial peptides indolicidin-OH, indolicidin-NH_2, bactenecin, polymyxin B, gramicidin D, and gramicidin S for 1 h at 30°C. Cell numbers were determined by the MPN method.

for 1 h, plated on *Desulfovibrio* agar (ATCC medium 42), and incubated in anaerobic GasPak chambers (Fisher Scientific Co, Pittsburgh, PA, USA).

Susceptibility of expression hosts to antimicrobial peptides

After 1 h exposure at 30°C, the viability of *B. subtilis* BE1500 was decreased 6000–10 000-fold by the purified acid form of indolicidin at 50–100 μg ml^{-1} and by only 100-fold by bactenecin at 50 μg ml^{-1}. Similarly, the viability of *B. subtilis* WB600 was decreased 20 000–40 000-fold by the purified acid form of indolicidin at 50–100 μg ml^{-1} and by only 400-fold by bactenecin (50 μg ml^{-1}).

Antimicrobial activity of indolicidin and bactenecin from recombinant Bacillus cultures against E. coli and D. vulgaris in batch suspension cultures and in biofilms

The ability of concentrated culture supernatant from *B. subtilis* BE1500 with the antimicrobial plasmids to kill *E. coli* BK6 and *D. vulgaris* was determined. No reduction in the viability of *E. coli* BK6 or *D. vulgaris* was observed for the negative-control experiments in which supernatants from *B. subtilis* BE1500(pBE92) were used (Table 1). However, the supernatant of *B. subtilis* BE1500(pBE92-Ind) with indolicidin inhibited *E. coli* BK6 by 12% and *D. vulgaris* by 38%. In addition, nearly 93% killing of *E. coli* BK6 and 90% killing of *D. vulgaris* was observed with supernatants from *B. subtilis* BE1500(pBE92-Bac) and *B. subtilis* BE1500(pBE92-ProBac). These results indicate that both indolicidin and bactenecin were expressed, secreted into the culture supernatant, and that the disulfide bond was processed properly in the extracellular environment to form cyclic active bactenecin. Furthermore, bactenecin is secreted at a concentration of approximately 3.0–3.5 μg ml^{-1} by *B. subtilis* BE1500(pBE92-Bac) based on a comparison with the killing of *E. coli* with purified bactencin [33] and on a comparison with the killing of *D. vulgaris* using purified bactenecin in this work (Figure 3).

The number of viable SRB after 5 days in a biofilm on 304 stainless steel in a non-shaken flask with *B. subtilis* BE1500 expressing the cloned antimicrobials was enumerated by the three-tube MPN assay (Table 2). Fewer SRB (36-fold) were present in the biofilm formed by *B. subtilis* BE1500(pBE92-Bac) compared with biofilms of the control *B. subtilis* BE1500(pBE92) (97% inhibition), while 10-fold less SRB was found with *B. subtilis* BE1500(pBE92-Probac) (90% inhibition). BE1500(pBE92-Ind) inhibited the SRB by 30% in the biofilm. Hence, secretion of the antimicrobials was also effective in inhibiting SRB in biofilms.

The ability of the antimicrobial-producing constructs to inhibit the growth of SRB on SAE 1018 mild steel in batch flasks was also studied. Upon addition of SRB (OD_{600} = 0.16–0.20) to a non-antimicrobial-producing *P. fragi* K culture, a strong odor of hydrogen sulfide was detected in less than 18 h. This was accompanied by formation of an iron sulfide black precipitate which indicates growth and colonization of SRB in the aerobic biofilm grown on the metal surface. *B. subtilis* BE1500 was capable of delaying the onset of SRB proliferation and increased corrosion by 36–48 h compared to *P. fragi* K, as evidenced by the delay of appearance of an iron sulfide precipitate and the odor of hydrogen sulfide. *B. subtilis* BE1500 with the three antimicrobial-producing constructs delayed the onset of SRB corrosion by an additional 96–120 h compared to *P. fragi* K and *B. subtilis* BE1500.

Continuous culture corrosion studies with Bacillus strains that produce a cloned antimicrobial peptide

The ability of the engineered *Bacillus* strains to inhibit SRB in biofilms by secreting antimicrobials was corroborated using electrochemical impedance spectroscopy (EIS) and 20 continuous fermentations (200–300 h). EIS is a non-invasive, *in situ* method that allows one to quantify accu-

Table 1 Susceptibility of *E. coli* BK6 and *D. vulgaris* to concentrated culture supernatants from *B. subtilis* BE1500 expressing antimicrobials[a]. Data shown were corroborated by a second, independent set of experiments

Source	*E. coli* BK6		*D. vulgaris*	
	CFU ml^{-1}	Inhibition (%)	MPN ml^{-1}	Inhibition (%)
Fresh medium	9×10^7	0	8.3×10^5	0
Buffer + kanamycin at 100 μg ml^{-1}	4×10^3	99.996	–	–
B. subtilis BE1500 (pBE92)	8.7×10^7	3	8.3×10^5	0
B. subtilis BE1500 (pBE92-Ind)	7.9×10^7	12	3.6×10^5	38
B. subtilis BE1500 (pBE92-Bac)	6.3×10^6	93	8.3×10^4	90
B. subtilis BE1500 (pBE92-ProBac)	7.2×10^6	92	8.8×10^4	89

[a]Incubation for 1 h at 30°C.

Table 2 Inhibition of SRB (determined by MPN) in an aerobic biofilm of *B. subtilis* BE1500 expressing the antimicrobial plasmids on 304 stainless steel after 5 days. Data shown were corroborated by a second, independent set of experiments

Plasmid	Inhibition (%)	Viable SRB (MPN ml^{-1})	Viable *B. subtilis* BE1500 (CFU ml^{-1})
pBE92	0	5.13×10^5	2.3×10^8
pBE92-Indolicidin	30	3.59×10^5	3.2×10^8
pBE92-Bactenecin	97	1.43×10^4	1.9×10^8
pBE92-Probactenecin	90	5.13×10^4	6.2×10^8

rately and sensitively the corrosion rate of metal [25,26]; hence, it allows one to study the impact of a biofilm on a metal surface without disturbing it [20]. Since the corrosion in these studies was induced by SRB in the biofilm, the reduction in corrosion measured was directly related to the decrease in the biofilm population of SRB.

Addition of SRB to a 304 stainless steel continuous reactor with non-antimicrobial-producing *P. fragi* K decreased the impedance at the lowest frequency measured (1.4×10^{-3} Hz) by 5-fold within 36 h of SRB addition. This decrease was also accompanied by the odor of hydrogen sulfide from the reactor outlet, and the reactor turned gray due to the formation of iron sulfide. The low frequency phase angle also decreased (80° *vs* 47°). A similar change in the impedance spectra was also observed with the negative control, *B. subtilis* WB600(pBE92); addition of SRB decreased the low frequency phase angle by 35° (Figure 4b). Correspondingly, the low-frequency impedance value (Z) also *decreased* by 7-fold (Figure 4a, note the log scale for the ordinate). This reduction in impedance (Z) indicates the corrosion rate *increased* 7-fold when SRB was added to the *B. subtilis* WB600(pBE92) biofilm since the low-frequency value of the impedance (resistance polarization, R_p) is *indirectly* proportional to the corrosion rate [23]. However, no decrease in impedance was observed upon addition of SRB with either the bactenecin-expressing or probactenecin-expressing *B. subtilis* WB600 biofilms which indicates there was no corresponding increase in corrosion (Figure 4). In addition, the phase angle curve was not shifted upon the addition of SRB for the *Bacillus* biofilms

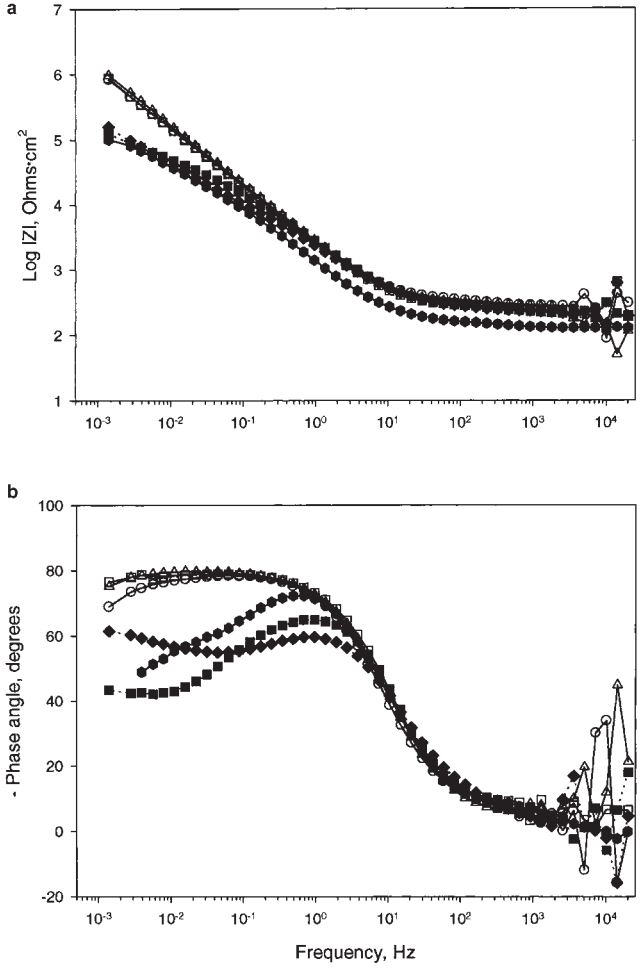

Figure 4 Representative impedance spectra of 304 stainless steel in modified Baars' medium with dual-cultures (a and b) of *B. subtilis* WB600 [with plasmid pBE92, pBE92-Ind (indolicidin), pBE92-Bac (bactenecin) or pBE92-ProBac (bactenecin with a pro-region)] and SRB *D. vulgaris*. –□– PBE92; --■-- pBE92 + SRB; --◆-- pBE92-Ind + SRB; –△– pBE92-Bac + SRB; –○– pBE92-ProBac + SRB; –●– *P. fragi* K + SRB.

expressing bactenecin or probactenecin. Therefore, these results indicate that growth of SRB on 304 stainless steel in the *B. subtilis* WB600(pBE92-Bac) and *B. subtilis* WB600(pBE92-ProBac) biofilms was inhibited significantly by the expression and secretion of bactenecin and

probactenecin. Along with these quantifiable indicators of SRB inhibition in biofilms when either bactenecin or probactenecin were expressed, there were also qualitative indicators of SRB inhibition in these fermentations such as a reduction in hydrogen sulfide production and a reduction in gray color compared to *B. subtilis* WB600(pBE92).

Expression of indolicidin in the biofilm did not inhibit SRB as well as bactenecin and probactenecin with WB600(pBE92-Ind). Upon addition of SRB, the low frequency phase angle decreased by 17° and the low-frequency impedance also decreased by 5.5-fold.

Similar EIS results were obtained with host *B. subtilis* BE1500 expressing the antimicrobials indolicidin, bactenecin, and probactenecin in 10 continuous fermentations (Figure 5), since the three antimicrobial-producing constructs were capable of decreasing the extent of change of the impedance spectra upon addition of SRB. The indolicidin construct was least effective in inhibiting SRB and the low frequency phase angle changed from 80° to 69°; however, the phase angle change was still less than that observed with the control pBE92 (80° to 61°). The bactenecin constructs (with and without the pro-region) were more effective than the indolicidin construct and the low frequency phase angle decreased only to 76°. Furthermore, expression of bactenecin and probactenecin in *B. subtilis* BE1500 decreased the corrosion rate by 6 to 12-fold (the low-frequency value of the impedance was not decreased upon SRB addition when these antimicrobials were expressed). These results indicated that the growth of SRB on 304 stainless steel had been inhibited significantly by the bactenecin constructs.

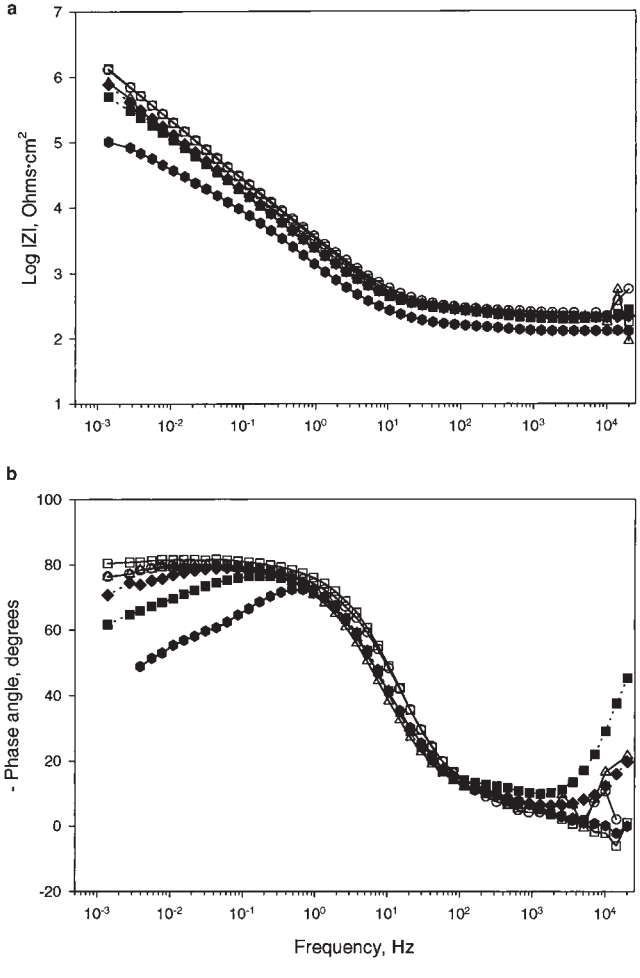

Figure 5 Representative impedance spectra of 304 stainless steel in modified Baars' medium with dual-cultures (a and b) of *B. subtilis* BE1500 [with plasmid pBE92, pBE92-Ind (indolicidin), pBE92-Bac (bactenecin) or pBE92-ProBac (bactenecin with a pro-region)] and SRB *D. vulgaris*. Symbols as in Figure 4. Data are from a representative experiment (two independent fermentations were conducted for each dual culture).

Discussion

These results indicate that peptide antimicrobials like gramicidin S, indolicidin, polymyxin B, and bactenecin have potential for use as inhibitors of the growth of SRB and can decrease microbially-influenced corrosion of steel. Amidated indolicidin is capable of inhibiting *Escherichia coli* and *Staphylococcus aureus* by 99.9% at 5–25 μg ml^{-1} [33,35], and in this study, *D. gigas* and *D. vulgaris* exhibited slightly greater resistance to amidated indolicidin (99.5% at 25–50 μg ml^{-1}, Figures 2 and 3). Bactenecin inhibits *E. coli* by 95% at 100 μg ml^{-1} [33] and demonstrated comparable inhibition of *D. gigas* and *D. vulgaris* (90%) in this study. Gramicidin S also completely inhibits growth of Gram-negative bacteria at 3–12.5 μg ml^{-1} [22] and inhibited both Gram-negative SRB in this study at 50–100 μg ml^{-1}. Based on their activity against SRB in suspension cultures, the antimicrobial peptides tested in this study seem to be more potent at comparable concentrations than commercially available antibiotics (eg, kanamycin, naladixic acid, tetracycline) and about as effective as sodium molybdate [1], but less effective than anthraquinone [10]; hence, these compounds may be attractive (less-toxic) antimicrobials for SRB.

While impregnating surfaces with antimicrobials [19] and releasing them based on a catalytic reaction to inhibit biofilm bacteria [39] may be applicable to medical devices, these techniques may not be cost-efficient in industrial settings where a wide range of materials with large surface areas are involved. Also, applications with large volumetric throughputs render chemical additives expensive. In such situations, it may be advantageous to use the *in situ* biofilm-population to generate antimicrobials from within the biofilm. The surrounding exopolysaccharide would also serve to prevent the antimicrobial from diffusing from the biofilm, thereby providing for increased local antimicrobial concentrations. This represents a general strategy that can be potentially applied in industrial or medical environments, irrespective of the substratum which the bacteria colonize.

Toward this goal, the cationic antimicrobial peptides indolicidin and bactenecin were expressed constitutively in *B. subtilis* BE1500 and WB600 as fusions to the signal peptide of the extracellular alkaline protease (Apr) by an approach similar to those used by Piers *et al* [30] and Pang *et al* [29]. The synthetic oligos for indolicidin and bactenecin were designed as precise fusions to the signal sequence so that no additional amino acids were added to the N terminus of the peptide.

The viability of *B. subtilis* was decreased by four-orders

of magnitude by the acid form of indolicidin. This suggests that *B. subtilis* would not be an ideal expression host for expressing indolicidin in biofilms, especially as indolicidin would not diffuse away as much as it would in suspension cultures and hence could attack the host cells. Protein engineering should lead to antimicrobials more specific for the targets and less specific for the host.

Continuous reactor experiments with 304 stainless steel demonstrated that growth of SRB was inhibited based on qualitative indicators like the reduction in odor of hydrogen sulfide and iron sulfide precipitate as well as changes in the impedance spectra and the quantitative lack of decrease in polarization resistance, R_p. The bactenecin constructs were more effective than the indolicidin construct in inhibiting the growth of SRB which suggests that bactenecin was expressed and processed properly to form a disulfide bond as defensins are usually inactive with improper disulfide bond processing [30]. Also, *B. subtilis* BE1500 and WB600 were more resistant to bactenecin than to indolicidin by a factor of 50–60 which could explain the enhanced ability of the bactenecin constructs to inhibit SRB on stainless steel. However, it was apparent that the SRB were not completely excluded from the biofilm as all reactors became more turbid upon the addition of SRB and a mild odor of sulfide was still detected from continuous reactors with *B. subtilis* BE1500(pBE92-Bac).

The current system demonstrates clearly that the growth of SRB on 304 stainless steel can be controlled by generating peptide antimicrobials from within the biofilm. Hence, there is potential for use of optimized bacterial systems in preventing microbiologically-influenced corrosion of steel (eg, cloning antimicrobial genes in bacteria better suited for growth in industrial settings). In addition, these techniques may also have applicability in preventing the growth of unwelcome bacteria in other environments (eg, combating infections).

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