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Role of SbmA in the Uptake of Peptide Nucleic Acid (PNA)-Peptide Conjugates in *E. coli*

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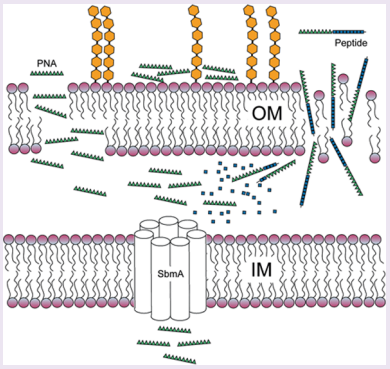
Supporting Information

ABSTRACT: Antisense PNA oligomers targeting essential genes (*acp*P or *fts*Z) and conjugated to the delivery peptide $L((KFF)_3K)$ show complete growth inhibition of wild type *E. coli* strain (MG1655) with submicromolar MIC. In this study we show that resistant mutants generated against such PNA-peptide conjugates had disruptions in the region of *sbm*A, a gene encoding an inner membrane peptide transporter. The wild type sensitivity to the PNA conjugates was re-established in the resistance mutants by complementation with *sbm*A. Furthermore, deletion of *sbm*A in *E. coli* AS19, a strain that is sensitive to unmodified PNA, resulted in resistance to PNA. Finally, PNA conjugated with the corresponding non-biological H-$D((KFF)_3K)$ peptide retained antibacterial activity in *sbm*A deletion strains, whereas the same conjugate with a protease-sensitive linker did not. These results clearly identify SbmA as a carrier of naked PNA over the inner bacterial membrane and thereby infer that the peptide is transporting the PNA conjugates over the outer membrane. Strains lacking SbmA were used to screen novel peptide-PNA carriers that were SbmA-independent. Four such PNA-peptide conjugates, H-$D((KFF)_3K)$, H-$(RFR)_4$-Ahx-βAla, H-$(R\text{-Ahx-}R)_4$-Ahx-βAla, and H-$(R\text{-Ahx})_6$-βAla, were identified that utilize an alternative uptake mechanism but retain their antimicrobial potency. In addition SbmA is the first protein identified to recognize PNA.



Multidrug resistance among pathogenic bacteria threatens to reverse gains made in human health over the past six decades. Indeed, for some infection indications only a single drug option exists,[1] with some pathogenic strains not responding to any of the front line options for treatment.[2] Antimicrobial resistance can spread horizontally between species, leading to multiple-drug resistance occurring rapidly in clinically significant pathogens. For instance, *Acinetobacter baylyi* is believed to have recently acquired >45 resistance genes from other species and has become a major cause of hospital-acquired infections worldwide.[3] Accordingly, major efforts are being devoted to identify novel bacterial targets as well as novel principles for antibacterial drug discovery, in the attempt to circumvent existing and wide spreading resistance mechanisms in the microbial fauna, in particular those relating to multidrug resistance against antibiotics in the clinic.[4,5]

Antisense technology has been exploited for more than 20 years in the discovery of highly specific drugs targeting selected genes at the mRNA level.[6] Subsequently, antisense agents were also proposed as future antibiotics, and proof of principle was obtained in *E. coli* using a pseudopeptide DNA mimic, peptide nucleic acid (PNA).[7,8] PNA targeted to the translation initiation region of an essential mRNA causes steric hindrance of ribosome binding, resulting in the inhibition of gene expression and cell death.[7] PNA entry into Gram-negative bacteria is limited by the outer-membrane lipopolysaccharide layer[9] and requires a carrier molecule in order to access the cell. Initially, the $L((KFF)_3K)$ peptide was identified as an efficient carrier,[8] and subsequently several arginine-rich, non-natural peptides have been described.[10,11]

Understanding the mode of action and resistance mechanisms of antimicrobial compounds is crucial in order to avoid rediscovery of known compounds, deselect compounds that kill nonselectively, predict clinical value, and develop second generation molecules that retain the activity of the parent but overcome known resistance mechanisms.[12] To date, there have been no reports of peptide-PNA-resistant bacteria. PNAs and peptide-PNA conjugates are not substrates for efflux pumps,[9,13] and thus likely resistance mechanisms could include enzymatic modification/degradation of the PNA or peptide carrier and sequence mutation at the mRNA target site or that of a transporter or "receptor" required for import. The aim of the study was to identify $L((KFF)_3K)$-PNA resistance mutants, determine the mechanism of resistance and use this mechanistic information to develop second generation peptide-PNAs. In this study we report, for the first time, the identification of peptide-PNA resistance mutants, through the discovery of the inner membrane protein SbmA as a transporter of naked PNA. Also we identify SbmA-independent antibacterial peptide-PNAs.

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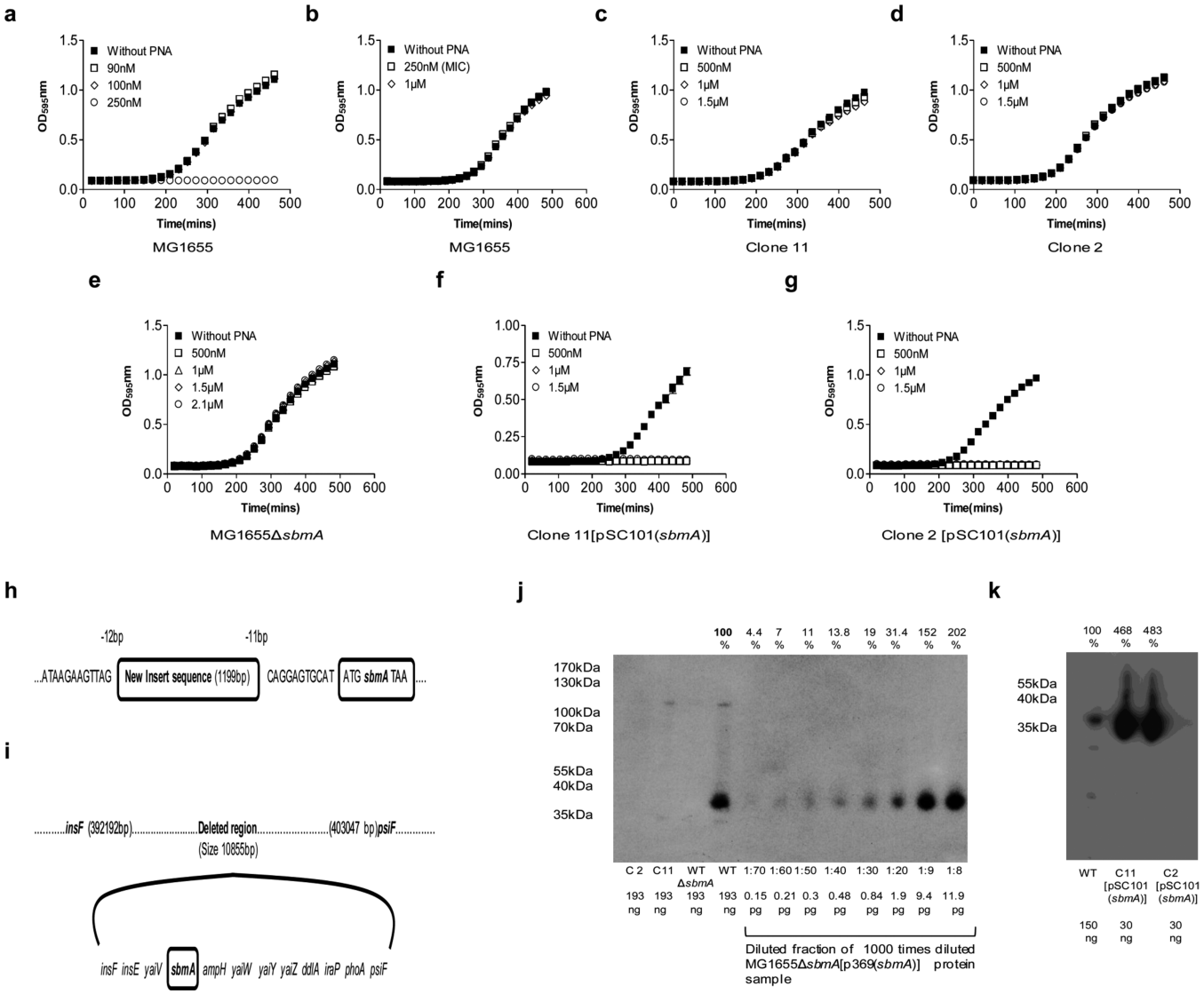

Figure 1. Activity of $L((KFF)_3K)$-PNA conjugate 2108 in various *E. coli* strains and clones. Dependence on SbmA protein level. (a, c–g) Dose-dependent effect of PNA 2108 on growth of MG1655, clone 11, clone 2, MG1655Δ*sbm*A, clone11[pSC101(*sbm*A)] and clone2[pSC101(*sbm*A)] strains. (b) Effect of the mismatch control PNA 3723 on growth of MG1655 strain. (h) Genomic structure of clone 11 around the region of the *sbm*A gene, having an insertion of 1199 bp upstream of the *sbm*A gene resembling the IS5 element (1195 bp). (i) Genome structure of clone 2 around the region of *sbm*A gene showing a deletion of 10,855 bp including 12 genes together with *sbm*A gene. (j, k) Quantification of the level of SbmA protein in MG1655 (WT), clone11, clone2, MG1655Δ*sbm*A, MG1655Δ*sbm*A [p369(*sbm*A)], clone 11[pSC101(*sbm*A)], and clone 2[pSC101(*sbm*A)] strains. Protein loading amount is indicated at the bottom of the blot.

RESULTS AND DISCUSSION

Identification of Mutants with Decreased Susceptibility to Peptide-PNAs. In this study we adopted a dual approach for the identification of *E. coli* mutants with decreased susceptibility to peptide-PNA conjugates: screening of single-gene mutants and *in vitro* selection by serial passage. PNAs targeting either of two well established antisense genes, *acp*P (PNA2108), which is essential for fatty acid synthesis, or *fts*Z (PNA2989), which is an essential component of the Z ring required in cell division, were used. The KEIO collection of 3,985 single gene deletion mutants was screened with PNA2108 (an anti *acp*P $L((KFF)_3K)$-PNA conjugate) at ×20 MIC (5 μM), in a high-throughput format. Two mutants, JW0368 (Δ*sbm*A) and JW5484 (Δ*ygh*F), showed significantly decreased susceptibility to the peptide-PNA (p = <0.001 and <0.01, respectively; see Supplementary Figure S1). Deletion of *sbm*A conferred complete resistance to PNA2108 up to ×20 MIC with growth being identical to that of the untreated control. Deletion of *ygh*F conferred partial resistance; growth occurred but with an approximate doubling of the lag phase. *In vitro* selection was done using PNA2108 and PNA2989 (an analogous anti *fts*Z $L((KFF)_3K)$-PNA conjugate) in separate experiments. Two mutants, clone 2 and clone 11, were identified that showed decreased susceptibility to the peptide-PNAs after 22 successive passages; both showed identical growth to untreated controls up to ×6 MIC (1.5 μM). The PNA target sites in clones 2 and 11 for both *acp*P and *fts*Z genes were sequenced, and both had DNA sequences identical to that of the parent strain, discounting mutation in the mRNA target sites for the PNAs as the mechanism responsible for the decreased susceptibility. As screening of single-gene deletion mutants had identified SbmA and YghF as proteins that were likely involved in peptide-PNA susceptibility, the DNA region

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Table 1. Antimicrobial Activity of PNA Peptide Conjugates

PNA no.	composition[a]	mass (calcd mass)	purity (%)[b]	gene target	MIC (μM)[c]						
					MG1655	Clone 11	Clone 2	MG1655 Δ*sbm*A	MG1655 [p369(*sbm*A)]	Clone 11 [p369(*sbm*A)]	Clone 2 [p369(*sbm*A)]
2108	H-KFFKFFKFFK-eg1-ctcatactct-NH_2	4176 (4178)	98	*acpP*	0.25	>1.5	>1.5	>2.1	0.1	0.5	0.5
3723	H-KFFKFFKFFK-eg1-ctcttacact-NH_2	4181 (4178)	98	mm	>1	ND	ND	ND	ND	ND	ND
2989	H-KFFKFFKFFK-eg1-ttcaaacatagt-NH_2	4797 (4792)	98	*ftsZ*	0.4	>1.5	>1.5	>2.1	0.2	0.5	0.5
3965	H-KFFKFFKFFK-eg1-ttctaacaaagt-NH_2	4794 (4792)	98	mm	>1	ND	ND	ND	ND	ND	ND
3759	H-D(KFFKFFKFK)-eg1-ctcatactt-NH_2	4168 (4178)	98	*acpP*	1	>2.1	1	1	0.9	>2.1	ND
3963	H-D(KFFKFFKFFK)-eg1-ctcttacact-NH_2	4181 (4178)	95	mm	>1.5	>2.1	>2.1	>2	ND	ND	ND
4201	H-D(KFFKFFKFFK)-FFK-eg1-ctcatactct-NH_2	4177 (4178)	98	*acpP*	1	ND	ND	>5	ND	ND	ND
4203	H-D(KFFKFFKFFK)-FFK-eg1-ctcttacact-NH_2	4381 (4382)	98	mm	>1.5	ND	ND	>5	ND	ND	ND
4202	H-D(KFFKFFKFFK)-FG-eg1-ctcatactct-NH_2	4178 (4178)	98	*acpP*	1	ND	ND	3	ND	ND	ND
4204	H-D(KFFKFFKFFK)-FG-eg1-ctcttacact-NH_2	4382 (4382)	98	mm	>1.5	ND	ND	5	ND	ND	ND
3756	H-RFRRFRRFRRFR-Ahx-(βAla)-ctcatactct-NH_2	4663 (4659)	95	*acpP*	1	>1.5	1.5	2.1	0.75	>1.5	0.75
4022	H-RFRRFRRFRRFR-Ahx-(βAla)-ctcttacact-NH_2	4660 (4659)	90	mm	>1.5	ND	>2	>2.1	ND	ND	ND
3757	H-RFRRFRRFRRFR-Ahx-(βAla)-tt aaacatagt-NH_2	5275 (5274)	90	*ftsZ*	1.5	>2.1	2.1	2.1	0.85	2.1	1.5
4023	H-RFRRFRRFRRFR-Ahx-(βAla)-ttctaacaaagt-NH_2	5273 (5274)	98	mm	>2	ND	>2.1	>2.1	ND	ND	ND
3986	H-(R-Ahx-R)$_4$-Ahx-(βAla)-ctcatactct-NH_2	4517 (4523)	90	*acpP*	0.2	0.4	0.2	0.2	ND	ND	ND
3987	H-(R-Ahx-R)$_4$-Ahx-(βAla)-ctcttacact-NH_2	4529 (4523)	90	mm	>1	>1	>1	>1	ND	ND	ND
4100	H-(R-Ahx-R)$_4$-Ahx-(βAla)-ttcaaacatagt-NH_2	5136 (5138)	98	*ftsZ*	0.5	2.1	0.5	0.5	ND	ND	ND
4102	H-(R-Ahx-R)$_4$-Ahx-(βAla)-ttctaacaaagt-NH_2	5130 (5138)	95	mm	>1	>1	>1	>1	ND	ND	ND
4099	H-(R-Ahx)$_6$-(βAla)-ctcatactct-NH_2	4324 (4324)	90	*acpP*	0.2	1	0.2	0.2	ND	ND	ND
4101	H-(R-Ahx)$_6$-(βAla)-ctcttacact-NH_2	4318 (4324)	90	mm	>1	>1	>1	>1	ND	ND	ND
3760	H-(R-Ahx)$_6$-(βAla)-ttcaaacatagt-NH_2	4931 (4939)	90	*ftsZ*	0.65	>2.1	0.75	0.65	0.65	>2.1	0.75
3964	H-(R-Ahx)$_6$-(βAla)-ttctaacaaagt-NH_2	4940 (4939)	95	mm	>1	ND	>1	>1	ND	ND	ND
2301	H-ctcatactct-NH_2	2638 (2637)	95	*acpP*	ND	ND	ND	ND	ND	ND	ND

[a]Amino acids are shown in uppercase letters, and PNA nucleobases in lowercase letters. The PNAs are written from their N- to C-termini, and the N-terminus. Ahx: 6-aminohexanoic acid; eg1: 8-amino-3,6-dioxaoctanoic acid. [b]98%, only minute contamination (very small peak); 95%, minor contamination (small size peaks); 90%, small contamination (foot and small size peaks). [c]MIC values are reported as the concentration resulting in an OD value <0.15 after 8 h of incubation at 37 °C. ND, not determined.

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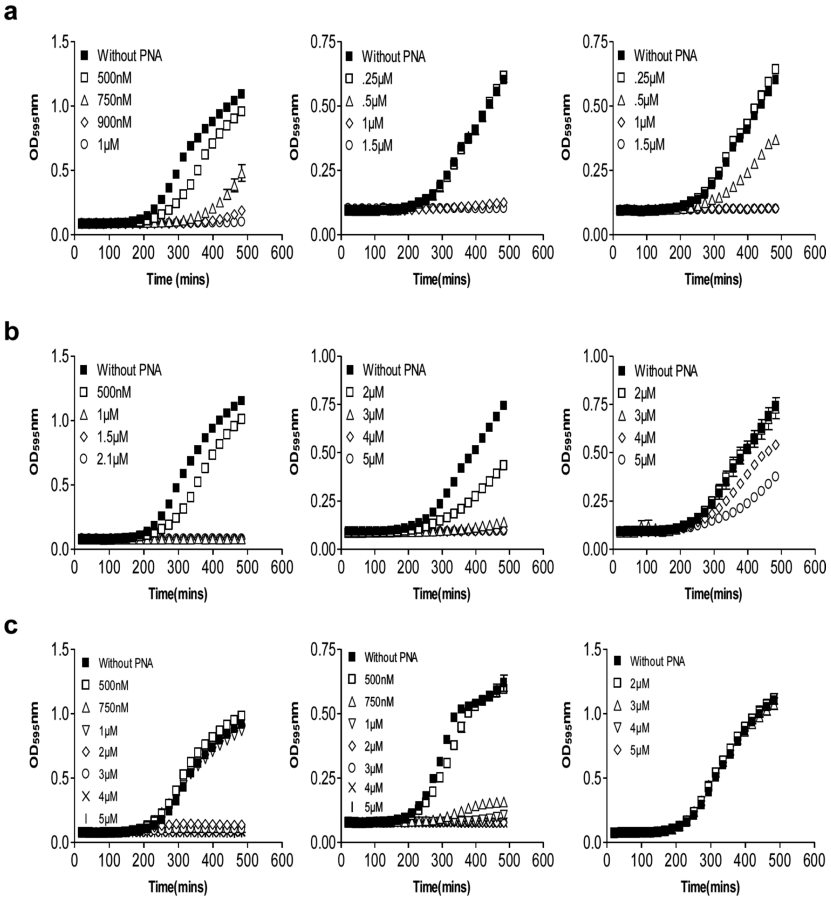

Figure 2. Bacterial growth inhibition of $D((KFF)_3K)$-PNA conjugate derivatives. (a, b) Dose effect of PNAs 3759, 4202, and 4201 (from left to right along the row) on the growth of MG1655 and MG1655Δ*sbm*A strain, respectively. PNA 4202 has -F-Gly-eg1- linker, while PNA 4201 possesses -FFK-eg1- linker in between PNA and the peptide (Table 1). (c) Dose effect of unmodified PNA 2301 on growth of outer membrane compromised strain AS19, AS19 [p369(*sbm*A)], and AS19Δ*sbm*A strains, respectively (from left to right along the row).

around both genes was investigated. Both mutants had intact copies of the *yghF* gene. Cloning and sequencing of the region surrounding *sbm*A revealed that clone 11 had an IS5 element (1195 bp)[14] inserted at the −11 bp site relative to the start codon. The IS5 element was in reverse orientation, relative to *sbm*A, and contained the following base pair changes 723 (C → G), 910 (C → T), 1157 (A → C), 1180 (C → A), 1184 (C → A), 1185 (T → G), and 1186 (T → A). Clone 2 had a 10,855 bp deletion in a region including *sbm*A and adjacent genes (*ins*F *ins*E, *yai*T, *yai*V, *amp*H, *sbm*A, *yai*W, *yai*Y *yai*Z, *ddl*A, *ira*P, *pho*A, and *psi*F) (Figure 1). The IS5 mobile element can provide *E. coli* with an opportunity to relieve stress conditions, either by activation of cryptic operons or inactivation of genes in response to exposure to toxic compounds.[15] The results from clone 2 and JW0368 (both *sbm*A deletion mutants) suggested that in clone 11 the IS5 element inactivates SbmA expression; this was confirmed by Western blotting, showing the absence of SbmA in all deletion strains (Figure 1j). To control for differences in the genetic backgrounds of *E. coli* MG1655, JW0368, and clones 2 and 11, an in-frame *sbm*A gene deletion mutant in *E. coli* MG1655 (MG1655Δ*sbm*A) was constructed. This strain had reduced susceptibility to both peptide-PNAs 2108 and 2989 (> ×20 MIC compared to the MG1655 parent). *E. coli* MG1655 and the Δ*sbm*A clones 2 and 11 were tested for their sensitivity to tetracyclin, ciprofloxacin, gentamicin, ampicillin, chloramphenicol, erythromycin, and nalidixic acid. The deletion mutants had identical MICs for all antibiotics except nalidixic acid where the MIC was ×1.6 that of MG1655 in both mutants (Supplementary Table S2).

Complementation of *sbm*A Gene Deletion Restores Susceptibility to the Peptide-PNAs. To confirm the role of SbmA in susceptibility toward the peptide-PNA, clones 2 and 11 as well as the *sbm*A deletion mutant were complemented with p369(*smb*A), a high-copy number plasmid expressing *sbm*A from the PBAD promoter, or PSC101(*smb*A), a low copy vector with *sbm*A under the control of its own promoter *sbm*A*p*. Complementation with either plasmids restored peptide-PNA susceptibility; overexpression of *sbm*A in strains harboring p369(*smb*A) increased susceptibility when compared to the parent strain MG1655 (Table 1). Complementation of *sbm*A deletion mutants was confirmed by Western blotting (see Figure 1k). The restoration of PNA susceptibility to wild-type levels, or higher (overexpression), indicates that SbmA alone is responsible for PNA uptake.

SbmA Is an Inner Membrane Transporter of PNA. SbmA is a 406 amino acid protein deduced to have seven transmembrane domains and is predicted to act as an importer.[16] Previous work has also demonstrated that SbmA is involved in transport of proline rich antimicrobial peptides,[17] and that *sbm*A mutants show complete resistance to the peptide antibiotics microcin B17 and MccJ25.[18] To determine whether the peptide, the PNA component and/or the entire peptide-PNA conjugate were the substrates of SbmA, we decided to study a series of alternative carrier peptides using either the D-

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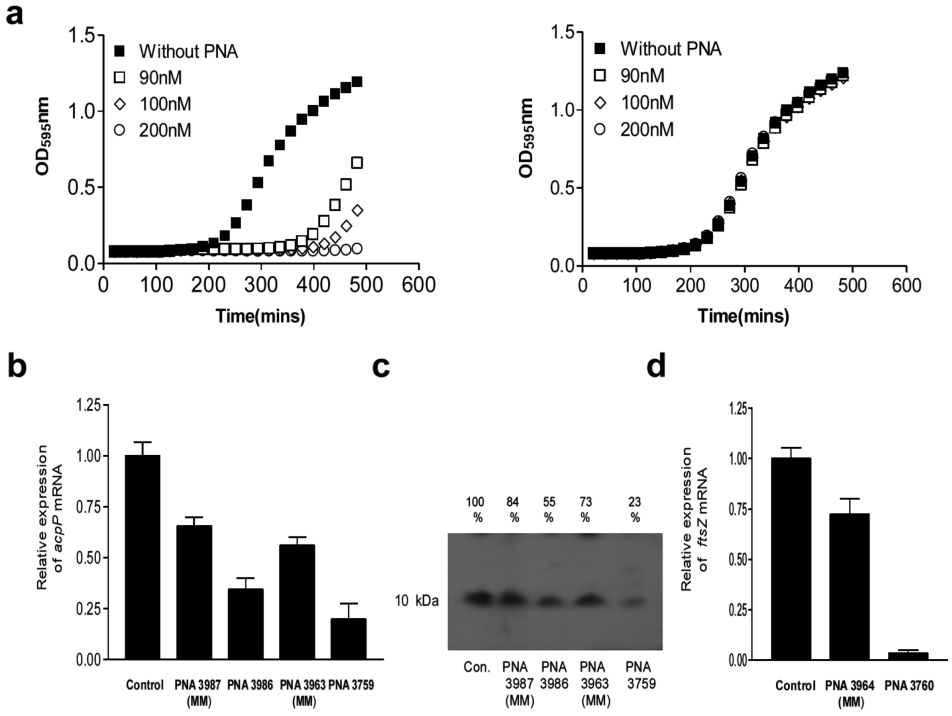

Figure 3. Characterization of novel peptide-PNA conjugates. (a) Dose-dependent effect of PNA 3986 and its mismatch control PNA 3987 (left to right) on growth of MG1655Δ*sbm*A. (b) Relative *acp*P mRNA abundance of *E. coli* MG1655 cells treated with PNAs 3986 and 3759 and their mismatch control (MM) PNAs. (c) AcpP quantification in cells treated as described in panel b. (d) Relative *fts*Z abundance for cells treated with PNA 3760 and its mismatch control.

form of the KFF peptide (PNA3759) or three arginine-based peptides, $(RFR)_4$ (PNAs 3756 and 3757); $(RAhxR)_4$ (PNAs 3986 and 4100), and $(RAhx)_6$ (PNAs 4099 and 3760), which were previously successfully used as carriers of PMO antisense agents.[10] Comparison of the sensitivity (in terms of MIC) of the wild type MG1655 strain and the corresponding Δ*sbm*A strain to these PNAs (Table 1) shows that deletion of *sbm*A only confers full resistance to the $L((KFF)_3K)$-PNA conjugates. These results indicate that the peptide plays a crucial role for the transport of the conjugate and that SbmA could have specificity for the $L((KFF)_3K)$ peptide but, as expected, not for the non-biological D-form of the same peptide. However, an alternative but at first sight maybe less likely explanation could be that it is indeed not the peptide but rather the PNA *per se* that is transported by SbmA. This would require that the penetration of the inner membrane by the $D((KFF)_3K)$-PNA (and by the arginine peptide conjugates) must occur *via* a different mechanism, and that the $L((KFF)_3K)$ peptide (but of course not the analogous D-peptide) is degraded by proteases in the periplasmic space. In order to test this hypothesis, we synthesized two additional $D((KFF)_3K)$-PNA derivatives having a short (protease-sensitive) L-peptide linker between the $D((KFF)_3K)$ and the PNA (FFK and FG for PNAs 4201 and 4202, respectively). These two conjugates exhibit identical activity toward wild type MG1655 (MIC = 1 μM) but, in contrast to the $D((KFF)_3K)$-PNA, are considerably less active in the Δ*sbm*A strains (MIC > 5 and 3 μM, respectively) (Table 1, Figure 2a,b). The highly reduced activity found for the corresponding mis-match control conjugates (PNA3963, 4203, and 4204) (Table 1) confirmed an antisense mechanism of action for the $D((KFF)_3K)$-type PNA conjugates. Consequently these data strongly corroborate the latter hypothesis. For further support we investigated *sbm*A deletion in *E. coli* AS19, a strain with a defective lipopolysaccharide (LPS) layer. PNAs usually require conjugation to a peptide carrier in order to cross the LPS layer of the outer membrane; however, unconjugated PNAs are active against *E. coli* AS19 while maintaining the antisense mode of action.[7] The MICs of naked (i.e., unconjugated) PNA 2301 (anti *acp*P) in strains *E. coli* AS19, *E. coli* AS19Δ*sbm*A, and *E. coli* AS19 [p369(*sbm*A)] were 2 μM, ≫5 μM, and 1 μM, respectively (Figure 2c). The dramatic decrease in PNA susceptibility in the Δ*sbm*A strain, together with the increase in susceptibility in the *sbm*A overexpression strain, is in agreement with results obtained for peptide-PNA conjugates in non-LPS defective strains and strongly supports the conclusion that SbmA is a transporter of naked PNA over the inner bacterial membrane.

Identification of Antibacterial Peptide-PNA Conjugants That Do Not Require SmbA for Activity. Prior understanding of resistance mechanisms that are likely to develop in the clinic is vital if candidate antibacterials are to progress into clinical application.[19] The above results clearly demonstrate the requirement of SbmA for antisense antibacterial PNAs that are conjugated to the $L((KFF)_3K)$ carrier peptide and predict that mutation in this transporter would be the most likely resistance mechanism to arise during exposure to such antibacterial PNAs. From both a mechanistic and antibacterial discovery point of view, identifying peptide-PNA resistance mutants was of interest as it enabled us to explore whether SbmA is required for PNAs conjugated to other carrier molecules, the aim being to discover antibacterial PNA conjugates that overcome the requirement for SbmA-mediated transport and thus would be active against Δ*sbm*A resistance mutants.

As already discussed above, the anti-*acp*P and *fts*Z PNAs conjugated to either of the other peptides used in this study

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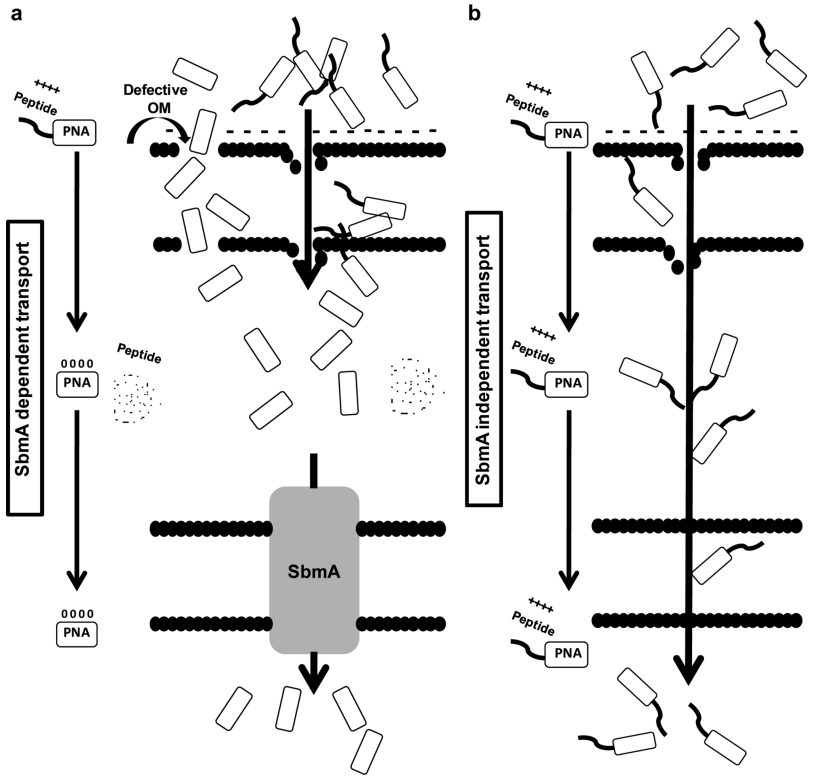

Figure 4. Schematic model of the cellular uptake of PNA and PNA-peptide conjugates. (a) The peptide-PNA conjugate crosses the outer membrane mediated by membrane "disturbance" by the peptide (in AS19 the PNA can cross without the aid of a peptide). Once in the periplasm, the peptide (*e.g.*, H-*L*((KFF)$_3$K) or H-*L*(RFR)$_4$) is degraded, and the free PNA crosses the inner membrane *via* transport by SbmA. (b) For peptide-PNA conjugates having a biostable peptide (*e.g.*, H-(R-Ahx)$_6$-*β*Ala, H-(R-Ahx-R)$_4$-Ahx- *β*Ala or H-*D*((KFF)$_3$K) uptake is exclusively taking place *via* peptide-mediated crossing of both the outer as well as the inner membrane.

(H-(RFR)$_4$-Ahx-*β*ala, H-*D*((KFF)$_3$K), H-(R-Ahx)$_6$-*β*ala, H-(R-Ahx-R)$_4$-Ahx-*β*ala) are active in *E. coli* Δ*sbm*A strains, and in particular the H-(R-Ahx)$_6$-*β*ala, H-(R-Ahx-R)$_4$-Ahx-*β*ala conjugates are as potent in this strain as the analogous *L*((KFF)$_3$K)-PNA conjugates are in the wild type (Table 1). Furthermore, these conjugates also show similar activity in the naturally selected resistant clone 2 (having a deletion of *sbm*A and adjacent genes), whereas highly reduced (or no) activity for all conjugates except PNA 3986 (the anti *acp*P H-(R-Ahx-R)$_4$-Ahx-*β*ala conjugate) is seen for clone 11 in which IS5 is inserted upstream of the *sbm*A gene. Thus in terms of general potency PNA3986 appears the most interesting of the conjugates, and we therefore decided to characterize this conjugate to confirm its antisense mode of action. As demonstrated by the experiments presented in Figure 3, growth is fully inhibited at 200 nM by the matched PNA, while a corresponding two base mismatch PNA (3987) had no effect on growth at this concentration (Figure 3a). As further evidence for a *bona fida* antisense effect, we measured the effect of both the match and the mismatch PNA on the level of target *acp*P mRNA (Figure 3b) as well as AcpP protein (Figure 3c); PNA 3986 significantly reduced mRNA as well as protein levels, while only slight (mRNA) or no effect (protein) was seen with the mismatch PNA3987. Similar differences between the effects of match and mismatch PNAs were also observed for the two other Δ*sbm*A active PNAs (3759 and 3760 (Figure 3b–d)). Furthermore, contrary to the anti *acp*P *L*((KFF)$_3$K)-PNA, screening of the KEIO collection with PNAs conjugated to H-(R-Ahx-R)$_4$-Ahx-*β*ala) (PNA3986) or H-(R-Ahx)$_6$-*β*ala (PNA4099) failed to identify resistance mutants. This indicates that these conjugates are not dependent on a single, nonessential protein for uptake.

Clone 2 and MG1655 Δ*sbm*A behave similarly in terms of peptide-PNA susceptibility (Table 1). Thus the additional gene deletions (apart from *sbm*A) in clone 2 do not seem to affect the sensitivity to the peptide-PNA conjugates. In contrast clone 11 had significantly higher MIC values for PNAs conjugated to the peptides H-(RFR)$_4$-Ahx-*β*ala, H-(R-Ahx-R)$_4$-Ahx-*β*Ala, and H-(R-Ahx-R)$_6$-*β*Ala when compared to both clone 2 and the MG1655 Δ*sbm*A strain (Table 1). The reason for this difference is not clear. The IS5 insertion in clone 11 clearly abolishes the expression of SbmA, but we do not know at this stage how the expression of other genes (downstream) is affected. It is also possible that there are, as yet, unidentified mutations in clone 11 that affect peptide-PNA sensitivity; future work will involve determining possible reasons for the increased resistance to peptide-PNAs in this strain.

The reason for the small, but significant, increase in resistance to *L*((KFF)$_3$K)-PNA in the *ygh*F deletion strain is not obvious. However, YghF is predicted to be a C-type secretion pathway protein. These proteins are thought to be located in the inner membrane and be part of the general signal-dependent export pathway, where they aid in the translocation of exoproteins from the cytoplasm to the periplasm. Homology detection and structural prediction[20] indicate that YghF has a protease domain. The partial resistance to *L*((KFF)$_3$K)-PNA conjugates conferred by deletion of *ygh*F may be due to it acting as a protease that aids in the degradation of the peptide carrier (see below for further details). Unlike *sbm*A deletion, which results in complete

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resistance to $L((KFF)_3K)$-PNAs, the predicted protease activity of YghF would presumably be partially complemented by other proteases present in the periplasm. Alternatively, YghF may be required for the secretion of proteins that facilitate uptake of peptide-PNAs across the outer membrane. Further characterization of YghF and its functional role in *E. coli* will aid in understanding its role in the uptake of peptide-PNAs (Figure 4).

Conclusions. The present results have identified the inner membrane transporter SbmA as an essential carrier of unmodified peptide nucleic acids and for peptide PNA conjugates in which the peptide is biodegradable. Furthermore, two spontaneous *E. coli* resistance mutants likewise mapped to the *sbm*A gene. As *sbm*A is nonessential and as orthologues are not present in all eubacterial species, the use of different carriers for different species may reduce the incidence of cross-resistance, *e.g.*, specific targeting of species with SbmA. Thus the alternative peptide carriers, described in this study, serve to broaden the spectrum of activity for PNA-based antimicrobials. As also demonstrated in this study a screen of novel antisense constructs in a Δ*sbm*A strain (if the target microbe harbors *sbm*A) is a good strategy to include in antibacterial PNA drug discovery programs.

Although only two mutant clones have been characterized so far, we find it noteworthy that resistance mutants having sequence changes in the antisense target were not isolated. This might indicate that resistance through such a mechanism may be less frequent than statistically expected, maybe due to the position of the target in a regulatory, noncoding part of the mRNA. We also note that both of the clones isolated are characterized by rather substantial genome changes (insertion and large deletion), which might negatively affect the virulence of the bacteria in a clinical situation.

Finally, in relation specifically to the biological properties of peptide nucleic acids, SbmA to the best of our knowledge represents the first example of biological recognition of this unnatural pseudopeptide by a natural protein (disregarding simple steric blocking of, *e.g.*, DNA polymerases or reverse transcriptases). This could reflect that SbmA may have evolved to recognize *inter alia* microcins, which are also a type of pseudopeptides, although their chemical structure is vastly different from that of PNA. Nonetheless, deciphering and understanding the structural elements of SbmA that presumably recognize PNA may from a chemical biology perspective allow the construction of synthetic proteins using PNA substrates, in search of, *e.g.*, PNases or ultimately even PNA polymerases.

METHODS

Peptide, PNA, and Peptide-PNA Conjugate Synthesis. All PNAs and peptide-PNAs used in the present study are listed in Table 1. Peptide, PNA, and peptide-PNA conjugates were synthesized by continuous solid phase synthesis using Boc-chemistry as previously described.[8,21] The conjugates were purified by reversed phase HPLC on an RP18 column using a 0−50% acetonitrile gradient in 0.1% TFA. Characterization in terms of purity and identity was done by HPLC and MALDI-TOF mass spectrometric analyses.

Bacterial Strains, Plasmids, Media, and Growth Conditions. All bacterial strains and plasmids used in the present study are listed in Supplementary Table 1. Bacterial cultures were grown in Luria broth (LB) or Mueller Hinton Broth (MHB) at 37 °C, with shaking (195 rpm) under aerobic conditions with the addition of antibiotics, when required, at the following concentrations: 100 μg mL^{-1} for ampicillin, 50 μg mL^{-1} for kanamycin, 15 μg mL^{-1} tetracyclin.

Minimum Inhibitory Concentration (MIC). MICs were determined as previously described.[7] Antimicrobial compounds were diluted in MHB by microdilution in a total volume of 250 μL in a microtiter plate. Wells were inoculated with approximately 5 log CFU mL^{-1} of cells and incubated with shaking (220 rpm) at 37 °C in a GENios microplate reader (Tecan) with continuous optical density (OD_{595}) measurement. MICs were defined as the lowest concentration of the antimicrobial compound that inhibited growth after 8 h of incubation.

High-Throughput Screening of the Single-Gene Deletion Mutants of *E. coli*. The Keio collection contains the single deletion mutants for all nonessential genes of *E. coli*.[22] The collection was subcultured into 96-well plates containing 200 μL of LB supplemented with kanamycin (LB/kan) and grown for 12 h at 37 °C with constant shaking (200 rpm). 1× plate bins were made by combining 10 μL of each strain, from a single Keio plate, into a microcentrifuge tube. Plate bins were concentrated by centrifugation (13,000 × *g*), washed, and resuspended in 500 μL of 15% (w/v) glycerol in LB/kan. 5× plate bins (mixes of 5 primary Keio collection plates) were made by subculturing 5 μL from each of five sequential 1× plate bins into 1 mL of LB/kan; growth and concentration were as above. All plate bins were frozen and stored at −80 °C until required. For peptide-PNA resistance screening, 5× plate bins were used as the inoculum for growth and MIC assays as described above. 5× bins that were positive for peptide-PNA resistance were further interrogated by screening of their constituent 1× bins. Such PNA-resistant 1× bins were then divided into column bins (as above). Finally, individual strains from PNA-resistant column bins were assayed.

***In Vitro* Selection for Decreased Susceptibility to Peptide-PNA.** *E. coli* MG1655 was the peptide-PNA susceptible parent, and peptide-PNAs 2108 (anti-*acp*P) and 2989 (anti-*fts*Z) were used in separate serial passage experiments. On day 1, approximately 7 log CFU mL^{-1} of exponentially growing cells were inoculated into 250 μL of 10% LB containing 2 μM of peptide-PNA. Cells were grown at 37 °C, with continuous shaking (220 rpm). Following overnight growth, cells were diluted to 7 log CFU mL^{-1} and used to inoculate the next day's assay, which was identical as above but with an increased peptide-PNA concentration. Serial passage experiments were continued for 22 days to a final peptide-PNA concentration of 5 μM. Individual colonies, recovered by plating onto LB agar, from the 22-day experiment, were purified by streak plating and were individually tested to determine the MIC of peptide-PNAs as above.

Cloning, Heterologous Expression, and Deletion of *sbm*A. Plasmids p369[23] and pSC101 (ATCC No. 37032) were used in the construction of strains for the heterologous expression of SbmA. The coding sequence of *sbm*A was amplified by PCR with primers containing restriction sites for directional cloning (see Supplementary Table S3 for a list of primers used in this study) yielding p369(*sbm*A) and pSC101(*sbm*A), respectively. Genomic DNA extraction, restriction digests, ligations, and transformation were done according to supplier's manuals, and plasmids were isolated using a Gene JET plasmid Mini Prep Kit (Fermentas Life Science). PCR was performed with 100 ng of template DNA or 0.5 μL of an overnight bacterial culture (colony PCR), 1 μM of each primer, 0.25 mM dNTPs, 2.5 μL of 10× ammonium buffer, and 0.5 μL of AccuPOL DNA polymerase, under the following conditions: 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 52−62 °C for 15 s, 72 °C for 30 s, followed by a final extension of 72 °C for 10 min. Post agarose gel electrophoresis on a 1% gel stained with ethidium bromide, PCR amplicons were purified using a Gene Jet PCR gel extraction purification kit according to the manufacturer's instructions. DNA sequencing of PCR amplicons and plasmids in order to validate plasmid constructs was done at Macrogen Inc. (Korea). SbmA expression in strains harboring p369(*sbm*A) was induced with 0.001% L-arabinose. Deletion of *sbm*A in *E. coli* strains MG1655 and AS19 by lambda RED-mediated recombination was done using a Quick and Easy *E. coli* Gene Deletion Kit (Gene Bridges) according to manufacturer's instructions. Deletion of *sbm*A was confirmed by PCR and sequencing

RNA Extraction, RT-PCR, and Real-Time PCR. Bacterial cells were grown in MHB for 6 h as above. RNA extraction was done using

a Pure Link RNA Mini Kit (Ambion). RNAprotect Bacteria Reagent (Qiagen) was used to prevent RNA degradation. On-column digestion was performed with PureLink DNase (Invitrogen), and gDNA contamination was checked by PCR. Maxima Reverse Transcriptase (Thermo Scientific) was used for cDNA conversion using gene specific primers (Supplementary Table S3) according to the manufacturer's instructions. Two microlitres of cDNA was amplified in 10 μL of PCR mix using 2 μL of LightCycler FastStart DNA Master^{PLUS} SYBR Green (Roche), 0.5 μM (0.1 μL) of each primer (Supplementary Table 1), and 5.8 μL of water. Reactions were performed in a Light Cycler (Roche) under the following conditions: an initial activation step at 95 °C for 10 min, 45 cycles of 95 °C for 10 s, 59 °C for 5 s, 72 °C for 30 s. Each reaction was performed in duplicate. Roche real-time software was used for data analysis and normalization. Data was taken from at least two independent biological samples.

Protein Detection and Quantification. Bacterial cells were grown in MHB or MHB supplemented with 0.001% L-arabinose for 6 h as above. Proteins, from both soluble and insoluble fractions, were extracted using the complete Lysis-B (2×) (Roche) or B-PER (Thermo Scientific) reagents according to manufacturer's instructions. Samples were separated on NuPAGE 4−12% Bis Tris gels (Life Technologies) and transferred to Hybond-P PVDF membranes (GE Healthcare). Purified proteins were detected by Western blotting with antibodies raised to AcpP or SbmA. Protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific).

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

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