Sequestration of Zinc Oxide by Fimbrial Designer Chelators

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Type 1 fimbriae are surface organelles of Escherichia coli. By engineering a structural component of the fimbriae, FimH, to display a random peptide library, we were able to isolate metal-chelating bacteria. A library consisting of 4 × 10^9 independent clones was screened for binding to ZnO. Sequences responsible for ZnO adherence were identified, and distinct binding motifs were characterized. The sequences selected exhibited various degrees of affinity and specificity towards ZnO. Competitive binding experiments revealed that the sequences recognized only the oxide form of Zn. Interestingly, one of the inserts exhibited significant homology to a specific sequence in a putative zinc-containing helicase, which suggests that searches such as this one may aid in identifying binding motifs in nature. The zinc-binding bacteria might have a use in detoxification of metal-polluted water.

In recent years a variety of expression systems for display of heterologous proteins on the surfaces of bacteria, bacteriophages, and yeasts have been developed (3, 11, 14). Many different types of surface proteins of gram-negative bacteria have been used to display foreign peptides: these surface proteins include LamB (4, 5, 22), flagellin (26, 29), Fim fimbriae (35, 38), OmpA (9), PhoE (1), peptidoglycan-associated lipoprotein (7, 10), OprI (6), Lpp (8), and type 1 fimbriae (13, 20, 30, 36). Enzymes, antigenic determinants, single-chain antibodies, metal-chelating peptides, and random peptide libraries have all been displayed on the surfaces of gram-negative bacteria. Systems in which random peptide libraries are expressed in connection with a surface protein have allowed screening of a vast number of peptides for binding to a certain target (11, 33). This technique allows construction of huge populations of diverse macromolecules and has been used in a number of different research fields. We are particularly interested in the display of heterologous peptides in type 1 fimbriae. A typical type 1 fimbriated bacterial cell has 200 to 500 peritrichous fimbriae on its surface. A single type 1 fimbria is a rod-shaped structure that is 7 nm wide and approximately 1 μm long and consists of four different components that are added to the base of the growing organelle (25). The bulk of the structure is made up of about 1,000 copies of the major subunit protein, FimA, polymerized into a right-handed helical structure, but small quantities of the minor components, FimF, FimG, and FimH, are also present (18, 24). It has been shown that the receptor-recognizing element of type 1 fimbriae is the 30-kDa FimH protein (23). The FimH protein is located at the tip and also is interspersed along the fimbrial shaft (16, 23). The FimF and FimG components are probably required for integration of the FimH adhesin into the fimbriae (16, 18).

The components of the fimbrial organelle are encoded by the chromosomal fim gene cluster (21). In addition to the structural components, this 9.5-kb DNA segment also encodes the fimbrial biosynthesis machinery, as well as regulatory elements (Fig. 1). The fimbrial organelle components, FimA, FimF, FimG, and FimH, are produced as precursors having N-terminal signal sequences. The N-terminal signal sequences are subsequently removed during export across the inner membrane. Thus, the FimH protein is produced as a 300-amino-acid precursor that is processed into a mature form containing 279 amino acids (12, 18). Further export from the periplasm across the outer membrane is dependent on a fimbria-specific export and assembly system made up of the FimC and FimD proteins (15, 17, 19).

In connection with the development of vaccine systems, heterologous sequences that represent immune-relevant sectors of proteins from foreign microorganisms have been authentically displayed on bacterial surfaces as fimbrial fusion proteins (30, 36). Previous work in our lab established that position 225 in FimH is a permissive site for insertion and display of heterologous sequences (30). This information was used to develop a system for display of random peptide libraries in Escherichia coli type 1 fimbriae. In an attempt to create a biological capture system for remediation of heavy metal contamination, we screened a peptide library for binding to ZnO. Here we describe the construction of ZnO-sequestering cells and the peptide sequences responsible for binding.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. In this study we used E. coli K-12 strain S1918 (F- lacI97 ΔmalB101 enaΔ laxr17 supE44 thiΔrelA gal96 ΔfimH-H-kan) (4). Cells were grown in Luria-Bertani medium supplemented with the appropriate antibiotics. Our FimH display system consisted of two plasmids, the FimH expression vector pLP30 and auxiliary plasmid pPKL115. Plasmid pLP30 is a pUC18 derivative containing the fimH gene downstream of the lac promoter. A BglII linker, located in a position corresponding to amino acid 225 (30), was used for integration of the random library. Plasmid pPKL115 is a pACYC184 derivative containing the whole fim gene cluster with a translational stop linker inserted in the fimH gene (30).

DNA techniques. Plasmid DNA was isolated by using a QIAprep Spin plasmid kit (QIAGEN). Restriction endonucleases were used as recommended by the manufacturer (Biolabs or Pharmacia). PCR to monitor the size and distribution of the random library were performed as previously described (36). The oligonucleotide primers used in these reactions were P1 (5'-CCTGACACGGGCGT CGGCGTAC) and P2 (5'-GGATAATTCTGACG) and were chemically synthesized (N indicates an equimolar mixture of all four nucleotides, and V indicates an equimolar mixture of A, C, and G). A primer oligonucleotide, 5'-ACTGTTGCTAGATC-3', was hybridized to the template oligonucleotide and extended with the Klenow fragment of DNA polymerase I. The double-stranded oligonucleotide was purified by phenol-chloroform extrac-
The resultant plasmid (pKKJ95) was indeed plasmid encoded. In addition, the ZnO-binding phenotype was indeed plasmid encoded. The primary DNA sequences encoding the binding peptides isolated from the random peptide library have been deposited in the GenBank database under accession no. AF167286 to AF167294.

**RESULTS**

Construction of random peptide library in FimH. We developed a binary plasmid system for displaying random peptides in the type 1 fimbrial adhesin, FimH. Chimeric FimH proteins displaying a random peptide library were engineered by using the FimH expression plasmid pLP30. This vector contains the *fimH* gene with an in-frame *BgII* linker inserted at a position corresponding to amino acid residue 225 of the mature protein and under transcriptional control of the inducible *lac* promoter (30).

The random library was constructed by inserting various numbers of synthetically synthesized 33-bp oligonucleotides into the *BgII* linker site. The presence of *BgII* overhangs in the linker resulted in an arginine-serine flanking sequence for each insertion. In addition, the presence of a VNN code in the random sequences ensured that functional stop codons were not present in an amber suppressing host. This genetic design allowed insertion of various numbers of double-stranded oligonucleotides into *fimH*, which increased the complexity of the library. In order to express the chimeric FimH proteins as functional constituents of type 1 fimbriae, we also used an auxiliary plasmid (pPKL115) which encodes the rest of the *fimH* gene cluster (Fig. 1).

Selection of ZnO-binding clones from the random library. Serial selection and enrichment of the random library was performed by using ZnO. To isolate cells adhering to ZnO particles, we used a 75% (vol/vol) Percoll solution which formed a density gradient upon centrifugation. Under these conditions only cells adhering to ZnO were able to sediment when they were centrifuged.

The genetic design of our random library allowed us to monitor the bacterial population by screening for changes in the number of inserts in *fimH* by PCR. Samples obtained after each enrichment cycle were analyzed by PCR amplification of the insert region by using primers complementary to the vector sequence flanking the insertion site (Fig. 1).

In a control experiment, in which neither Percoll nor metal oxide was added, no perturbation in the distribution of insert sizes was observed during the enrichment procedure (Fig. 2A).

However, a PCR analysis of the population after four cycles of enrichment with ZnO revealed that representation of clones having inserts consisting of one and two oligonucleotides increased (Fig. 2B). Cells obtained from the fourth enrichment cycle were spread onto agar plates, and cultures were established from 40 single colonies. The abilities of cells expressing the enriched peptides to adhere to ZnO were examined by phase-contrast microscopy. The behavior of one clone and the behavior of a control strain expressing wild-type FimH are shown in Figure 3A and B. Approximately 63% (25 of 40) of the clones selected exhibited a ZnO-binding phenotype and were examined further.

The *fimH*-containing plasmids were purified and individually retransformed into S1918 (pPKL115) cells. The reconstructed clones exhibited the same capacity to adhere to ZnO as the original isolates, indicating that the ZnO-binding phenotype was indeed plasmid encoded. In addition, the ZnO-binding capacities of these clones were not affected when Percoll and methyl-α-D-mannopyranoside were removed from the M63 salts medium. The agglutination titers of the cells were similar to the agglutination titer of a control strain expressing wild-type FimH.

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type FimH, indicating that the inserts did not influence the natural binding domain of FimH or significantly alter the number of fimbriae on the surface of the cells.

**Analysis of isolated sequences.** The insert regions in *fimH* of 25 individual ZnO-binding clones were sequenced, and we identified 11 different sequences which could confer on *E. coli* cells the ability to bind to ZnO (Fig. 4A). The insert sequences of pJKS9, pJKS11, pJKS12, and pJKS10 were represented eight, four, three, and two times, respectively, in these preparations. Most of the clones had two inserts, which is consistent with the PCR results obtained from monitoring the insert distribution. Interestingly, one of the two inserts in pJKS18 was also found in pJKS15, which provided strong evidence that this sequence plays a role in ZnO binding.

A number of structural similarities were discerned when we examined the amino acid sequences enriched for binding to ZnO. The three different binding motifs from the enriched amino acid sequences are underlined (RX2RS), double underlined (PXRS), and italicized (R/T-HXK-D/Q). The boldface letters represent amino acids encoded by the BglII linkers.

**Quantification of binding with enriched sequences.** The presence of the insert sequence from plasmid pJKS9 in 8 of the 25 clones suggested that the sequences which we selected may exhibit different ZnO-binding avidities. In order to investigate this possibility, bacteria associated with ZnO particles were counted directly. Our enumeration of bound cells revealed differences in avidity among the ZnO-binding clones examined (Fig. 5). The strongest binding capacity was observed with cells harboring plasmid pJKS9, which indicated that the FimH display system was able to enrich for sequences with different degrees of affinity for ZnO.

**Identification of a zinc-binding motif in a putative helicase.** The isolated sequences in the random library were compared to sequences in the SWISS-PROT database. Interestingly, a zinc-binding motif was identified in a putative helicase. The isolated sequences in the random library were compared to sequences in the SWISS-PROT database.
database search revealed a striking similarity (level of identity, 63%) between the insert of pJKS9 and a 24-amino-acid portion of a putative helicase from \( S. \) pombe (Fig. 6). The level of sequence similarity was 50% if the arginine-serine pairs encoding the \( BgII \) sites was not taken into consideration. Since helicases are generally known to be zinc-containing enzymes involved in separation and unwinding of DNA double helixes (34), we hypothesized that this helicase sequence might be involved in zinc binding.

To examine this possibility, two oligonucleotides encoding the identified motif in the putative helicase were annealed and ligated into pLPA30. The resultant plasmid (pKKJ95) was then transformed into S1918(pPKL115). Cells expressing the sequence motif in FimH were observed to bind to ZnO (Fig. 3C).

The binding strength of the putative helicase motif was quantified by counting ZnO-associated bacteria (Fig. 5). Cells harboring pKKJ95 exhibited a binding strength similar to that of the ZnO-binding clone enriched from the random peptide library (pJKS9). These results suggest that metal-binding sequences enriched with the FimH display system may be used to elucidate similar motifs in naturally occurring proteins.

**Characterization of the binding specificities of selected sequences.** In order to determine the binding specificities of the sequences enriched for binding to ZnO, we examined their abilities to adhere to CdO. Zinc and cadmium are metals that are closely related as determined by various chemical criteria, as expected on the basis of their close proximity in the periodic table of the elements. Interestingly, we found that whereas some of our clones did not exhibit affinity for CdO, about 50% of the clones did indeed bind to this metal (Fig. 3 and 5). Neither of the two closely related insert sequences in plasmids pJKS9 and pKKJ95 conferred an ability to adhere to CdO. Thus, our system could enrich for clones with different binding affinities (i.e., one subset of clones that could adhere only to ZnO and a second subset of clones that could bind to both ZnO and CdO). We also examined the affinities of the clones for different forms of zinc. To do this, we performed two sets of experiments. In the first set, different amounts of ZnCl\(_2\) (a soluble form of zinc) were preincubated with the clones before we tested for binding to ZnO, and this treatment had no effect on the ability of the clones to coordinate ZnO. However, it should be noted that at the highest concentration of ZnCl\(_2\), used the cells lysed, which prevented us from conducting inhibition experiments with concentrations higher than 2 mM. In the second set of experiments we monitored the abilities of our clones to bind to nitritotriacetic acid-chelated Zn\(^{2+}\) resin. Consistent with the results of the previous experiments, no affinity for Zn\(^{2+}\) was observed, which indicated that the binding was highly specific for the oxide form of zinc.

**DISCUSSION**

Accumulation of heavy metals in the environment due to human activities in the biosphere has been a growing threat to soil, groundwater, and public health over the last few decades. Zinc is an important trace metal in many biological processes (37); however, at higher concentrations it is a biological hazard. The spread and accumulation of zinc in the environment are due to the many uses of this metal; it is used in alloys, electroplating, electronics, automotive parts, fungicides, roofing, cable wrapping, and nutrition and health care products. Previously, it has been suggested that the ability of bacteria to sequester and immobilize heavy metals could be used to develop a biological method for remediation of heavy-metal-polluted wastewater (22, 27, 31).

We developed a system for displaying a random peptide library in connection with the FimH adhesin of \( E. \) coli type 1 fimbriae. A serial enrichment process was used, and peptide sequences conferring the ability to coordinate ZnO were selected from the random library and characterized. We observed a number of common structural characteristics in the amino acid sequences selected. The oligonucleotide encoding the amino acid sequence YDRSMPH was identified independently in plasmids pJKS15 and pJKS18, which suggested that at least part of this sequence is involved in coordination of ZnO. In addition, three different sequence motifs were discerned in the enriched sequences. In some cases these sequences were associated with the arginine-serine linker sequence incorporated into the genetic design of the random library. The ability to coordinate ZnO, however, was not strictly dependent on the presence of these motifs as other sequences were also selected during the enrichment procedure.

Taking into account the VNN design of the random library, we enriched the amino acids histidine, methionine, aspartate, and arginine to various degrees in the ZnO-binding sequences. Significantly, all of these amino acids have been previously observed to participate in metal-protein interactions (2). Histidine is known to be associated with the coordination of Zn\(^{2+}\) in zinc fingers and metalloproteins (37), and the charged radicals in aspartate and arginine could perhaps be involved in the chelation of zinc. A sequence lacking histidine (pJKS10) was also isolated, which indicated that histidine is not absolutely required for adherence to ZnO.

In a previous study, Barbas et al. (2) identified peptide sequences that conferred the ability to coordinate Zn\(^{2+}\) in a phage-displayed semisynthetic combinatorial antibody library. Only 50% of the peptides identified contained histidine, which indicated that the presence of histidine in Zn\(^{2+}\)-binding peptides is not required. We did not observe any similarities between the Zn\(^{2+}\)-binding sequences identified by Barbas et al. (2) and the ZnO-binding sequences identified in this study. However, this could be explained simply by the fact that the metal targets were different in the two studies. In addition, a number of other factors intrinsic to the type of display system, such as the genetic structure of the random library, the buffers, the selection and enrichment procedure employed, and the flanking protein sequences, are known to affect the type of sequences enriched when these techniques are used.

Direct counting of cells of various clones revealed differences in the number of ZnO-binding cells, which indicated that the FimH display system allows selection of peptide sequences with a variety of binding avidities. In particular, one clone (pJKS9) exhibited a high affinity for ZnO compared to the other clones isolated. Furthermore, this sequence exhibited a remarkably high degree of similarity to a putative helicase motif found during a database search. Many helicases are known to be zinc-containing enzymes, and cloning of the putative helicase motif in FimH resulted in ZnO-binding cells, suggesting that this motif is indeed involved in zinc binding. These findings suggest that the technology described here might be used to identify binding domains in naturally occurring proteins.

Some of the ZnO-binding clones isolated also exhibited an
affinity for CdO. Given the chemical relatedness of Zn and Cd, this result is not surprising. However, the presence of clones that adhered to ZnO but not to CdO indicates that metal-specific binding sequences can be identified by the procedures used. We are currently investigating this aspect of the process in order to better understand the rules governing metal-protein interactions.

Bacterial surface display of designer chelators in connection with type 1 fimbriae is a powerful system for engineering bacteria with biosorptive abilities. We can envisage that a heterobinary adhesin suitable for the development of biomatrices could be designed by using the natural d-mannose-binding domain of FimH in combination with a second engineered site. In this context the techniques described here may become valuable tools for capturing or immobilizing metal pollutants.

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REFERENCES