Biotinylation Facilitates the Uptake of Large Peptides by *Escherichia coli* and Other Gram-Negative Bacteria

Jennifer R. Walker¹ and Elliot Altman^{2*}

Department of Microbiology¹ and Center for Molecular BioEngineering, Department of Biological and Agricultural Engineering,² University of Georgia, Athens, Georgia

Received 16 January 2004/Accepted 27 October 2004

Gram-negative bacteria such as *Escherichia coli* can normally only take up small peptides less than 650 Da, or five to six amino acids, in size. We have found that biotinylated peptides up to 31 amino acids in length can be taken up by *E. coli* and that uptake is dependent on the biotin transporter. Uptake could be competitively inhibited by free biotin or avidin and blocked by the protonophore carbonyl *m*-chlorophenylhydrazine and was abolished in *E. coli* mutants that lacked the biotin transporter. Biotinylated peptides could be used to supplement the growth of a biotin auxotroph, and the transported peptides were shown to be localized to the cytoplasm in cell fractionation experiments. The uptake of biotinylated peptides was also demonstrated for two other gram-negative bacteria, *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa*. This finding may make it possible to create new peptide antibiotics that can be used against gram-negative pathogens. Researchers have used various moieties to cause the illicit transport of compounds in bacteria, and this study demonstrates the illicit transport of the largest known compound to date.

The outer membrane of gram-negative bacteria functions as a molecular sieve and allows only very small molecules to passively diffuse into the cell. Porins in the outer membrane allow the transport of larger molecules and may be specific or nonspecific in their molecular recognition. Nonspecific porins such as OmpF, OmpC, and PhoE allow the rapid passage of hydrophilic molecules (27, 28). Other porins allow the transport of specific molecules. The peptide permeases, for example, have a specificity for oligopeptides. The uptake of oligopeptides is dependent upon size, hydrophobicity, and charge (5, 26, 32).

It is well documented that *Escherichia coli* cannot take up large peptides and that the size exclusion limit for porin-mediated peptide transport is 650 Da or the size of a penta- or hexapeptide (31, 33). The size exclusion limit for peptide uptake in other gram-negative organisms such as *Salmonella enterica* serovar Typhimurium has also been determined and found to be similar to that in *E. coli* (31, 33). In contrast to gram-negative bacteria, gram-positive bacteria can transport much larger peptides. For example, *Lactococcus lactis* has been shown to take up peptides more than 18 residues in length or 2,140 Da in size (10) while *Bacillus megaterium* can transport molecules up to 10,000 Da in size (40).

This study provides evidence that large biotinylated peptides can be readily transported into gram-negative bacteria such as *E. coli*. While conducting an in vivo screen for randomly encoded peptides that could inhibit the growth of *Staphylococcus aureus*, we performed a test to confirm that potential peptides resulting from the screen would be readily taken up, as expected, by this gram-positive organism. A biotinylated 10-amino-acid peptide was added extracellularly to growing cultures

of *S. aureus* and an *E. coli* control, which should not have been able to take up the 1,534-Da peptide. The synthetic peptides had been biotinylated so they could be easily visualized on Western blots with a NeutrAvidin horseradish peroxidase conjugate. Surprisingly, we found that the peptide was taken up by both *S. aureus* and *E. coli* within 5 min of incubation. This observation appeared to contradict the known size exclusion limit of *E. coli* and suggested that biotinylation of peptides may allow peptide uptake to occur via the biotin transport system.

MATERIALS AND METHODS

Bacterial strains. *E. coli* MG1655 (wild type F[−] λ[−]), *E. coli* S1036 (Δ bio-61 bioP98 [up promoter] *recA1 thi rpsL* λ b515 b519 *galQ6 red-270 cI857*), *E. coli* S1039 [*birB13*(Ts) Δ bio-61 bioP98 (up promoter) *recA1 thi rpsL* λ b515 b519 *galQ6 red-270 cI857*], *E. coli* SA291 [*rpsL his* Δ (*gal-chlA*)], *Pseudomonas aeruginosa* ATCC 9721, *S. enterica* serovar Typhimurium LT2, and *S. aureus* ATCC 25923 were the bacterial strains used in this study. *E. coli* S1036 and S1039 were derived from SK121, which is a derivative of SK98 (18) and contains a mutation in the λ prophage that enables SK121 to grow at 43°C (Allan Campbell, personal communication).

Medium. Rich LB and minimal M9 medium as described by Miller (22) were used for *E. coli* MG1655 and *S. enterica* serovar Typhimurium cultures. Rich LB and minimal medium as described by Gilleland et al. (14) were used for *P. aeruginosa*. Tryptic soy broth and minimal medium as described by Mah et al. (21) were used for *S. aureus*. Rich LB and minimal medium as described by Campbell (7) were used for *E. coli* S1036, S1039, and SA291. Glucose was the carbon source used in the minimal medium for the uptake experiments, except for the fractionation studies, in which maltose was used instead.

Peptides and reagents. The randomized biotinylated peptides XXXX(KBt-n)XXXXA (10 amino acids) and XXXXXXXXXX(KBt-n)XXXXXX XXXXXXXXA (31 amino acids) were synthesized by Sigma Genosys, where A denotes the L-amino acid alanine, X denotes an equimolar mixture of all 20 natural L-amino acids, and KBt-n denotes the L-amino acid lysine to which biotin has been attached. The average molecular masses of the 10- and 31-amino-acid peptides were determined to be 1,534 and 3,904 Da, respectively, with an Applied Biosystems Voyager System 1105 mass spectrometer. This was in very close agreement with the theoretical molecular masses of the 10- and 31-amino-acid peptides, which were 1,517 and 3,947 Da, respectively. Biotin, thiamine, avidin, and bovine serum albumin were purchased from Sigma. NeutrAvidin horseradish peroxidase conjugate and SuperSignal West Dura extended-duration chemiluminescent substrate were purchased from Pierce.

* Corresponding author. Mailing address: Center for Molecular BioEngineering, Department of Biological and Agricultural, Driftmier Building, University of Georgia, Athens, GA 30602. Phone: (706) 542-2900. Fax: (706) 542-8806. E-mail: ealtman@uga.edu.

Uptake assays. Minimal 37°C overnight cultures were diluted into fresh minimal medium and incubated at 37°C until they reached an optical density at 550 nm (OD_{550}) of 0.5. The 10- and 31-amino-acid randomized biotinylated peptides were added to the medium at a concentration of 1 μ g/ml of culture. After addition of the peptide to the culture, 1-ml aliquots were extracted at intervals of up to 1 h, washed twice of extracellular peptide with fresh minimal medium, and then boiled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gradient sample buffer. Samples were run on a 10 to 16% Tricine gradient gel (39) and transferred to nitrocellulose membranes. The resulting Western blots were treated with NeutrAvidin horseradish peroxidase conjugate and SuperSignal West Dura extended-duration chemiluminescent substrate. The membranes were incubated for 5 to 10 min and then exposed to X-ray film for 1 to 10 min. Bands on the film were quantified with the AlphaEase 5.5 densitometry program from Alpha Innotech.

To test the effects that biotin, thiamine, avidin, bovine serum albumin, and carbonyl *m*-chlorophenylhydrazine (CCCP) had on peptide uptake, these compounds were added to mid-log-phase cultures 5 min before the addition of the biotinylated peptide. One-milliliter samples were extracted 10 min after the addition of the peptide and analyzed by SDS-PAGE as described above.

An upper 22,500-Da protein band can be seen in the Western blots involving *E. coli* samples that are shown in Fig. 1, 2, 3, 4, 5, and 7. This is the *E. coli* biotin carboxyl carrier protein, which is the prominent biotinylated protein in *E. coli* (12). Multiple upper bands can be seen in the Western blots involving *S. enterica* serovar Typhimurium and *P. aeruginosa* samples that are shown in Fig. 8. Most bacteria contain several biotinylated proteins, and the multiple biotinylated bands shown in the Western blots involving *S. enterica* serovar Typhimurium and *P. aeruginosa* are consistent with this fact. Additional protein bands ranging from 22,500 to 4,000 Da can be seen in the blots involving *E. coli* samples that are shown in Fig. 3 and 7. These two blots were exposed to film longer than the other blots that are shown in Fig. 1, 2, 4, 5, and 7, and these extra bands are likely extraneous background bands that appear because of overdevelopment of the blot. The biotinylated peptides in Fig. 1 and 8 disappear overtime. This is due to degradation by peptidases and proteases that are present in bacterial cells (43). All studies were done in triplicate; however, only one representative Western blot is shown for each experiment.

Cell fractionation. The 31-amino-acid biotinylated peptide was added to *E. coli* MG1655 cells that had been grown to an OD_{550} of 0.5 in minimal maltose medium to allow induction of the maltose binding protein, which served as one of the fractionation controls. After an additional 10 min of incubation, the cultures were subjected to periplasmic shock as described by Ames et al. (3) to isolate the periplasmic fraction. The remaining cell pellet was then further fractionated as described by Altman et al. (1) to prepare cytoplasmic and membrane fractions, with one modification. Cytoplasmic proteins were precipitated by adding trichloroacetic acid at a final concentration of 5% (wt/vol) to the cytoplasmic fraction. The precipitate was then centrifuged at 4°C and 50,000 rpm for 30 min in a Beckman Ti 70.1 rotor to pellet the cytoplasmic proteins. The periplasmic, cytoplasmic, and membrane samples were analyzed with a 10 to 16% Tricine gradient gel and Western blotted as described above for the uptake assays.

RESULTS

Biotinylated peptides up to 31 amino acids in length can be taken up by *E. coli*. We initially tested the ability of *E. coli* and *S. aureus* to import a 10-amino-acid biotinylated peptide. Randomized peptides were used as opposed to peptides with a specific sequence in order to avoid nonspecific uptake that might be caused by certain amino acid sequences. Peptide was added to mid-log-phase cultures of bacteria that were incubated for up to 60 min. Samples were removed at specific times, pelleted, washed to remove any peptide in the medium that had not been taken up by the cells, and then analyzed as described in Materials and Methods. As shown in Fig. 1, both *E. coli* and *S. aureus* readily imported the 10-amino-acid biotinylated peptide. Using densitometry, we determined that up to 75% of the peptide was imported within the first 5 min of incubation. To determine whether the import, which was arguably due to biotinylation in *E. coli*, was limited to smaller peptides, we also tested whether a much larger 31-amino-acid

FIG. 1. Uptake of a 10-amino-acid (aa) biotinylated peptide by *S. aureus* (A) and *E. coli* MG1655 (B). The biotinylated peptide was added to mid-log-phase cultures, and samples were taken at different time intervals and analyzed by SDS-PAGE as described in Materials and Methods. Peptide-only and cell-only samples were included as controls.

biotinylated peptide could be imported into *E. coli* and *S. aureus*. As with the 10-amino-acid biotinylated peptide, the 31-amino-acid biotinylated peptide was also taken up by both *E. coli* and *S. aureus* (data not shown).

Uptake of biotinylated peptides in *E. coli* can be competitively inhibited by biotin or avidin and blocked by the protonophore CCCP. Given that peptides larger than six amino acids cannot be taken up by *E. coli*, the obvious interpretation of our results was that biotin transport was the mechanism by which this unexpected uptake was occurring. To test this assumption, we conducted a competition experiment in both *E. coli* and *S. aureus* with biotin. We rationalized that since large peptides can be readily taken up by gram-positive bacteria such as *S. aureus*, biotin should have no competitive effect. However, in *E. coli*, if the uptake was due to biotin, then free biotin should be able to competitively block uptake. Figure 2 shows that this is indeed the case. The uptake of biotinylated peptides could be blocked in *E. coli* by the addition of biotin, whereas biotin had no effect on the uptake of biotinylated peptides in *S. aureus*.

Additionally, we showed that the competitive inhibition in *E.*

FIG. 2. Effect of biotin on the uptake of a 31-amino-acid (aa) biotinylated peptide in *E. coli* MG1655 and *S. aureus*. Biotinylated peptide and equimolar or 10 $\times$ equimolar amounts of biotin or thiamine were added to mid-log-phase cultures. The cell samples were processed and analyzed by SDS-PAGE as described in Materials and Methods.

coli was specific to biotin and the use of another similarly sized vitamin, thiamine, had no effect. Because avidin is known to tightly bind biotin (15), we also tested whether avidin would be able to competitively inhibit the uptake of biotinylated peptides in *E. coli*. Figure 3 shows that avidin could competitively inhibit the uptake of biotinylated peptides in *E. coli* but that the use of another similarly sized protein, bovine serum albumin, which is routinely used in *in vitro* studies, had no effect.

It has been shown that biotin uptake is blocked by the protonophore CCCP, which disrupts membrane potential in *E. coli* (34, 35). If the uptake of biotinylated peptides was due to the biotin transport system, then CCCP would be expected to block the uptake of biotinylated peptides. Figure 4 shows that uptake is blocked when CCCP is added prior to addition of the biotinylated peptide.

The uptake of biotinylated peptides in *E. coli* is dependent on the biotin transport system. The biotin transport system in *E. coli* has been well characterized, and mutations that prevent the uptake of biotin, the *birB* and *bioP* mutations, are available (9, 11). If the import of biotinylated peptides in *E. coli* were indeed due to the biotin transport system, then *birB* mutants should not be able to take up biotinylated peptides. Figure 5

FIG. 3. Effect of avidin on the uptake of a 31-amino-acid (aa) biotinylated peptide in *E. coli* MG1655. Biotinylated peptide and equimolar or 10 $\times$ equimolar amounts of avidin or bovine serum albumin (BSA) were added to mid-log-phase cultures. The cell samples were processed and analyzed by SDS-PAGE as described in Materials and Methods.

FIG. 4. Effect of CCCP on the uptake of a 31-amino-acid (aa) biotinylated peptide in *E. coli* MG1655. CCCP was added at a final concentration of 50 μ M to mid-log-phase cultures of MG1655 since it has been shown that *E. coli* continues to grow normally at this concentration of CCCP (19). The cell samples were processed and analyzed by SDS-PAGE as described in Materials and Methods.

shows that this is the case. A *birB*⁺ wild-type strain was able to take up biotinylated peptide, while an isogenic *birB* mutant strain was not.

Biotinylated peptides can be used to fulfill the growth requirements of an *E. coli* biotin auxotroph. To further demonstrate that biotinylated peptides were truly taken up by *E. coli*, we tested whether a biotinylated peptide could be used instead of biotin to fulfill the growth requirement of an *E. coli* biotin auxotroph in minimal medium. Figure 6 shows that an *E. coli* biotin auxotroph grows as well in medium supplemented with biotinylated peptide as it does in medium supplemented with biotin.

Cell fractionation studies show that the biotinylated peptide can be detected in the cytoplasm of *E. coli*. To demonstrate biochemically that biotinylated peptides were taken up by *E. coli*, we performed cell fractionation studies in which periplasmic, cytosolic, and membrane fractions were prepared from cultures to which biotinylated peptide had been added. Figure

FIG. 5. Effect of a *birB* mutation on the uptake of a 31-amino-acid (aa) biotinylated peptide in *E. coli*. The biotinylated peptide was added to mid-log-phase cultures of *birB*⁺ and *birB* mutant cells. After 15 or 30 min of incubation, cell samples were processed and analyzed by SDS-PAGE as described in Materials and Methods. Peptide-only and cell-only samples were included as controls.

FIG. 6. Growth of an *E. coli* *bio* auxotroph on minimal medium supplemented with biotin or equimolar amounts of biotinylated peptides. The *E. coli* SA291 *bio* auxotrophic strain was grown in minimal medium at 37°C with either no supplement (Δ), 1 µg of biotin per ml (□), or equimolar amounts of the 10 (○)- and 31 (◇)-amino-acid biotinylated peptides. Aliquots were removed at 12-h intervals, and the OD₅₅₀ was determined.

7 shows that the biotinylated peptide localized to both the cytoplasmic and membrane fractions. Of the peptide that could be detected, 66% was found in the membrane fraction and 34% was found in the cytoplasmic fraction. To verify that the cell fractionation studies had been done correctly, we used the same cell fractions to visualize the GroEL and MBP proteins,

FIG. 7. Localization of the biotinylated peptide in *E. coli*. Biotinylated peptide was added to mid-log-phase cultures of MG1655, and the cells were fractionated into periplasmic, cytoplasmic, and membrane samples and analyzed by SDS-PAGE as described in Materials and Methods. Peptide-only and whole-cell-plus-peptide samples were included as controls. aa, amino acids.

FIG. 8. Uptake of a 31-amino-acid (aa) biotinylated peptide by *S. enterica* serovar Typhimurium (A) and *P. aeruginosa* (B). Biotinylated peptide was added to mid-log-phase cultures, and aliquots were taken at different time intervals and analyzed by SDS-PAGE as described in Materials and Methods. Peptide-only and cell-only samples were included as controls.

which are known to localize to the cytoplasm and periplasm, respectively. GroEL was found primarily in the cytoplasmic fraction, while MBP was found primarily in the periplasmic fraction. GroEL's distribution was 93% in the cytoplasm and 7% in the membrane, while MBP's distribution was 95% in the periplasm, 3% in the membrane, and 2% in the cytoplasm (data not shown).

Biotinylated peptides can be taken up by other gram-negative bacteria. Given our findings obtained with *E. coli*, we also wanted to test whether biotinylated peptides could be transported by other gram-negative bacteria. We found that both the 10- and 31-amino-acid biotinylated peptides could be readily transported by both *S. enterica* serovar Typhimurium and *P. aeruginosa*. Figure 8 shows the uptake of the 31-amino-acid biotinylated peptide by *S. enterica* serovar Typhimurium and *P. aeruginosa*.

DISCUSSION

It has been well established that gram-negative bacteria such as *E. coli* can only take up very small peptides that are six

amino acids or less in size. In this study, we have shown that biotinylation can facilitate the uptake of peptides up to 31 amino acids in length by *E. coli* and that transport is dependent on the biotin transporter, BirB or BioP. We have found that uptake of biotinylated peptides can be competitively inhibited by free biotin or avidin and blocked by the protonophore CCCP, which disrupts membrane potential. We also demonstrated that biotinylated peptide could be used to supplement the growth of a biotin auxotroph and that the biotinylated peptide was localized to the cytoplasm in cell fractionation studies. What is known about biotin function in *E. coli* is consistent with our finding that biotin can be used to facilitate the uptake of peptides via the biotin transporter in *E. coli*.

Biotin can be synthesized as well as transported by *E. coli*, and the genes involved in biotin biosynthesis and transport are repressible by biotin (16). Biotin's transport system is regulated independently of the biosynthetic pathway (29). *E. coli* readily imports the vitamin when it is available and concomitantly represses biotin synthesis. Biotin uptake is specific and energy dependent and can accumulate against a concentration gradient (34, 35, 36). Maximum uptake is observed during the exponential growth phase (34), and glucose has been shown to increase biotin uptake slightly. The rate of biotin uptake has also been shown to increase in proportion to the amount of extracellular biotin that is available (36).

The first mutation that affected biotin transport was discovered by Campbell et al. (8). They termed the mutation *bir* for biotin retention and showed that the mutation abolished the ability of *E. coli* to take up biotin. Eisenberg et al. (11) isolated an independent mutation that abolished biotin uptake, which they termed *bioP*. Campbell et al. (9) renamed their original *bir* mutation *birB* and showed that the *birB* and *bioP* mutations are identical via genetic mapping experiments.

It is surprising that the biotin transport system can be used to facilitate the uptake of large peptides. Biotin has a molecular weight of 244, making it relatively small in comparison to a 10-amino-acid biotinylated peptide with an average molecular weight of 1,534 or a 31-amino-acid biotinylated peptide with an average molecular weight of 3,904. The biotin uptake system must be flexible since it can accommodate larger molecules. Our finding that 34% of the biotinylated peptide localized to the cytoplasm and 66% of the peptide localized to the membrane is consistent with such a model. Some of the biotinylated peptide was able to completely pass through the biotin transporter, while a significant fraction remained in the membrane.

There is contradictory evidence about how biotin's structure affects its ability to be taken up by *E. coli*. Prakash and Eisenberg (36) stated that while the ureido ring of biotin must be intact for uptake, modification of the side chain has little effect. However, Piffeteau et al. (34) suggested that modifications to the side chain of biotin could drastically affect biotin's ability to be transported and that the carboxyl group on the side chain is essential for biotin uptake. In the biotinylated peptides used in this study, the biotin carboxyl group is joined to the amino group of lysine via an amide bond and thus the carboxyl group of biotin is not available for recognition. This fact supports Prakash and Eisenberg's argument that the side chain of biotin does not affect uptake. Our data further suggest that it is

indeed the ureido ring that is required for recognition and uptake.

The fact that biotinylation can facilitate the uptake of very large peptides by gram-negative bacteria represents the illicit transport of the largest known compound to date. Illicit transport has been defined as the entry of compounds into cells through the use of transport systems designed for other substrates (2). There are numerous examples of the use of peptide permeases to facilitate the uptake of small antibacterial peptides or antibiotics that have been coupled to di- or tripeptides (2, 4, 13, 25, 42). Additionally, researchers have used various siderophores that are involved in iron uptake to facilitate the transport of antibiotics (20, 23, 44). All of these compounds are much smaller than the 10- and 31-amino-acid peptides that we have found to be transported via biotinylation.

Interestingly, biotinylated molecules are currently being investigated for drug delivery in mammalian cells. Avidin drugs that bind to biotinylated vectors are being used to promote delivery across the blood-brain barrier (6, 30, 41), while anti-tumor toxins or imaging agents coupled to streptavidin are being delivered with biotinylated antibodies (17, 37). Biotinylation has also been shown to promote the delivery of polyethylene glycol-camptothecin conjugates into human ovarian carcinoma cells (24) and increase the cellular uptake of polyethylene glycol-TAT nonapeptide conjugates into human Caco and CHO cells (38).

Our finding that biotinylated peptides can be taken up by gram-negative bacteria such as *E. coli*, *S. enterica* serovar Typhimurium, and *P. aeruginosa* represents an intriguing possibility for the development of antibacterial peptides. Given the abundance of naturally occurring antibacterial peptides and the increased interest in designing new synthetic peptide drugs, researchers have been trying to develop novel peptide antibiotics that can inhibit the function of key intracellular targets identified through genomics. Researchers have been focusing on gram-positive bacteria, where the uptake of large peptides is not problematic. The use of biotinylated peptides may make it possible to use this same approach to develop antibacterial peptides that can target gram-negative bacteria.

ACKNOWLEDGMENTS

This work was supported by grants from the University of Georgia Biotechnology Awards Program and Zolaris BioSciences.

We thank Allan Campbell for providing the isogenic *birB*⁺ and *birB* mutant *E. coli* strains.

REFERENCES

- Altman, E., R. K. Altman, J. M. Garrett, R. J. Grimaila, and R. Young. 1983. *S* gene product: identification and membrane localization of a lysis control protein. *J. Bacteriol.* **155**:1130–1137.
- Ames, B. N., G. F.-L. Ames, J. D. Young, D. Tsuchiya, and J. Lecocq. 1973. Illicit transport: the oligopeptide permease. *Proc. Natl. Acad. Sci. USA* **70**:456–458.
- Ames, G. F.-L., C. Prody, and S. Kustu. 1984. Simple, rapid, and quantitative release of periplasmic proteins by chloroform. *J. Bacteriol.* **160**:1181–1183.
- Atherton, F. R., M. J. Hall, C. H. Hassall, S. W. Holmes, R. W. Lambert, W. J. Lloyd, and P. S. Ringrose. 1980. Phosphonopeptide antibacterial agents related to alafosfalin: design, synthesis, and structure-activity relationships. *Antimicrob. Agents Chemother.* **18**:897–905.
- Barak, Z., and C. Gilvarg. 1975. Specialized peptide transport system in *Escherichia coli*. *J. Bacteriol.* **122**:1200–1207.
- Bonfils, E., C. Mendes, A. C. Roche, M. Monsigny, and P. Midoux. 1992. Uptake by macrophages of a biotinylated oligo- α -deoxythymidylate by using mannosylated streptavidin. *Bioconjug. Chem.* **3**:277–284.
- Campbell, A. 1961. Sensitive mutants of bacteriophage λ . *Virology* **14**:22–32.

8. Campbell, A., A. del Campillo-Campbell, and R. Chang. 1972. A mutant of *Escherichia coli* that requires high concentrations of biotin. *Proc. Natl. Acad. Sci. USA* **69**:676–680.
9. Campbell, A., R. Chang, D. Barker, and G. Ketner. 1980. Biotin regulatory (*bir*) mutations of *Escherichia coli*. *J. Bacteriol.* **142**:1025–1028.
10. Detmers, F. J. M., E. R. S. Kunji, F. C. Lanfermeijer, B. Poolman, and W. N. Konings. 1998. Kinetics and specificity of peptide uptake by the oligopeptide transport system of *Lactococcus lactis*. *Biochemistry* **37**:16671–16679.
11. Eisenberg, M. A., B. Mee, O. M. Prakash, and M. R. Eisenberg. 1975. Properties of α -dehydrobiotin-resistant mutants of *Escherichia coli* K-12. *J. Bacteriol.* **122**:66–72.
12. Fall, R. R., A. W. Alberts, and P. R. Vagelos. 1975. Analysis of bacterial biotin-proteins. *Biochim. Biophys. Acta* **379**:496–503.
13. Fickel, T. E., and C. Gilvarg. 1973. Transport of impermeant substances in *E. coli* by way of oligopeptide permease. *Nat. New Biol.* **241**:161–163.
14. Gilleland, H. E., Jr., J. D. Stinnet, and R. G. Eagon. 1974. Ultrastructural and chemical alteration of the cell envelope of *Pseudomonas aeruginosa*, associated with resistance to ethylenediaminetetraacetate resulting from growth in a Mg^{2+} -deficient medium. *J. Bacteriol.* **117**:302–311.
15. Green, N. M. 1963. Avidin. The nature of the biotin-binding site. *Biochem. J.* **89**:599–609.
16. Guha, A. 1971. Divergent orientation of transcription from the biotin locus of *Escherichia coli*. *J. Mol. Biol.* **56**:53–62.
17. Hussey, S. L., and B. R. Peterson. 2002. Efficient delivery of streptavidin to mammalian cells: clathrin-mediated endocytosis regulated by a synthetic ligand. *J. Am. Chem. Soc.* **124**:6265–6273.
18. Ketner, G., and A. Campbell. 1975. A deletion mutation placing the galactokinase gene of *Escherichia coli* under control of the biotin promoter. *Proc. Natl. Acad. Sci. USA* **72**:2698–2702.
19. Kinoshita, N., T. Unemoto, and H. Kobayashi. 1984. Proton motive force is not obligatory for growth of *Escherichia coli*. *J. Bacteriol.* **160**:1074–1077.
20. Luckey, M., J. R. Pollack, R. Wayne, B. N. Ames, and J. B. Neilands. 1972. Iron uptake in *Salmonella typhimurium*: utilization of exogenous siderochromes as iron carriers. *J. Bacteriol.* **111**:731–738.
21. Mah, R. A., D. Y. C. Fung, and S. A. Morse. 1967. Nutritional requirements of *Staphylococcus aureus* S-6. *Appl. Microbiol.* **15**:866–870.
22. Miller, J. H. 1972. Experiments in molecular genetics. Cold Springs Harbor Laboratory, Cold Springs Harbor, N.Y.
23. Miller, M. J., J. A. McKee, A. A. Minnick, and E. K. Dolence. 1991. The design, synthesis and study of siderophore-antibiotic conjugates. *Biol. Met.* **4**:62–69.
24. Minko, T., P. V. Paranjpe, B. Qiu, A. Lalloo, R. Won, S. Stein, and P. J. Sinko. 2002. Enhancing the anticancer efficacy of camptothecin using biotinylated poly(ethylene glycol) conjugates in sensitive and multidrug-resistant human ovarian carcinoma cells. *Cancer Chemother. Pharmacol.* **50**:143–150.
25. Morely, J. S., T. D. Hennessey, and J. W. Payne. 1983. Backbone-modified analogues of small peptides: transport and antibacterial activity. *Biochem. Soc. Trans.* **11**:798–800.
26. Naider, F., and J. M. Becker. 1975. Multiplicity of oligopeptide transport systems in *Escherichia coli*. *J. Bacteriol.* **122**:1208–1215.
27. Nikaïdo, H. 1996. Outer membrane, p. 29–47. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
28. Nikaïdo, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.* **49**:1–32.
29. Pai, C. H. 1973. Biotin uptake in biotin regulatory mutant of *Escherichia coli*. *J. Bacteriol.* **116**:494–496.
30. Pardridge, W. M. 2002. Targeting neurotherapeutic agents through blood-brain barrier. *Arch. Neurol.* **59**:35–40.
31. Payne, J. W. 1980. Transport and utilization of peptides by bacteria, p. 211–256. In J. W. Payne (ed.), *Microorganisms and nitrogen sources*. John Wiley & Sons, Inc., New York, N.Y.
32. Payne, J. W., and C. Gilvarg. 1968. Size restriction on peptide utilization in *Escherichia coli*. *J. Biol. Chem.* **243**:6219–6299.
33. Payne, J. W., and M. W. Smith. 1994. Peptide transport by micro-organisms. *Adv. Microb. Physiol.* **36**:1–80.
34. Piffeteau, A., M. Zamboni, and M. Gaudry. 1982. Biotin transport by a biotin-deficient strain of *Escherichia coli*. *Biochim. Biophys. Acta* **688**:29–36.
35. Piffeteau, A., and M. Gaudry. 1985. Biotin uptake: influx, efflux, and countertransport in *Escherichia coli*. *Biochim. Biophys. Acta* **816**:77–82.
36. Prakash, O. M., and M. A. Eisenberg. 1974. Active transport of biotin in *Escherichia coli* K-12. *J. Bacteriol.* **120**:785–791.
37. Press, O. W., M. Cocoran, K. Subbiah, D. K. Hamlin, D. S. Wilbur, T. Johnson, L. Theodore, E. Yau, R. Mallett, D. L. Meyer, and D. Axworthy. 2001. A comparative evaluation of conventional and pretargeted radioimmunotherapy of CD20-expressing lymphoma xenografts. *Blood* **98**:2535–2543.
38. Ramanathan, S., B. Qiu, S. Pooyan, G. Zhang, S. Stein, M. J. Leibowitz, and P. J. Sinko. 2001. Targeted PEG-based bioconjugates enhance the cellular uptake and transport of a HIV-1 TAT nonapeptide. *J. Control. Release* **77**:199–212.
39. Schägger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1–100 kDa. *Anal. Biochem.* **166**:368–379.
40. Scherrer, R., and P. Gerhardt. 1971. Molecular sieving by the *Bacillus megaterium* cell wall and protoplast. *J. Bacteriol.* **107**:718–735.
41. Song, B.-W., H. V. Vinters, D. Wu, and W. M. Pardridge. 2002. Enhanced neuroprotective effects of basic fibroblast growth factor in regional brain ischemia after conjugation to a blood-brain delivery vector. *J. Pharmacol. Exp. Ther.* **301**:605–610.
42. Staskawicz, B. J., and N. J. Panopoulos. 1980. Phaseolotoxin transport in *Escherichia coli* and *Salmonella typhimurium* via the oligopeptide permease. *J. Bacteriol.* **142**:474–479.
43. Walker, J. R., R. K. Altman, J. Warren, and E. Altman. 2003. Using protein-based motifs to stabilize peptides. *J. Peptide Res.* **62**:214–226.
44. Wittmann, S., M. Schnabelrauch, I. Scherlitz-Hofmann, U. Mollmann, D. Ankel-Fuchs, and L. Heinisch. 2002. New synthetic siderophores and their β -lactam conjugates based on diamino acids and dipeptides. *Bioorg. Med. Chem.* **10**:1659–1670.