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Uptake of Cell Wall Peptides by *Salmonella typhimurium* and *Escherichia coli*

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During bacterial growth, cell wall peptides are released from the murein and reused for the synthesis of new cell wall material. Mutants defective in peptide transport were unable to reutilize cell wall peptides, demonstrating that these peptides are taken up intact into the cytoplasm prior to reincorporation into murein. Furthermore, cell wall peptide recycling was shown to play an important physiological role; peptide transport mutants which were unable to recycle these peptides showed growth defects under appropriate conditions. Using mutants specifically defective in each of the three peptide transport systems, we showed that the uptake of cell wall peptides was mediated solely by the oligopeptide permease (Opp) and that neither the dipeptide permease (Dpp) nor the tripeptide permease (Tpp) played a significant role in this process. Our data indicate that the periplasmic oligopeptide-binding protein has more than one substrate-binding site, each with different though overlapping specificities.

Gram-negative bacteria such as *Salmonella typhimurium* and *Escherichia coli* are able to utilize a wide variety of small peptides as nutrients (16). As these species do not secrete peptidases, peptides are taken up intact and only hydrolyzed intracellularly. Three genetically distinct permeases, with overlapping substrate specificities, serve to mediate the uptake of peptides into cytoplasm (6). The dipeptide permease (Dpp) is relatively specific for dipeptides and also provides the chemoreceptor for peptide chemotaxis (6, 14). The tripeptide permease (Tpp) has a restricted specificity, with the highest affinity for hydrophobic tripeptides (1a). Expression of the *tppB* gene, which encodes the tripeptide permease, is only induced during anaerobic growth (11, 12) and depends upon the function of the positive regulatory proteins OmpR and EnvZ (1); the physiological role of this transport system is not yet understood. The third peptide uptake system, the oligopeptide permease (Opp), provides the major pathway for the uptake of small peptides consisting of up to five amino acid residues. Opp is a binding-protein-dependent transport system and has been extensively characterized (4, 7–10). Mutants deficient in all three of these permeases show no detectable uptake of any peptide tested (6). Thus, if any additional peptide transport system is present, it must be specific for a restricted number of peptides.

Recently, it has been shown that *E. coli* can reutilize peptides which are normally released from the murein cell wall during growth (2, 3). Some of these peptides escape from the periplasm into the growth medium, but most (80 to 90%) are recycled. The two major cell wall peptides, L-Ala-D-Gly-γ-*meso*-diaminopimelic acid (L-Ala-D-Glu-γ-*m*-A_2pm) and L-Ala-D-Gly-γ-*m*-A_2pm-D-Ala, differ from protein-derived peptides in that they contain D-amino acids and a γ-peptide bond. It has previously been reported that the peptide permeases show a strong preference for protein-derived peptides containing L-amino acids and α-peptide bonds; synthetic peptides containing D-amino acids or modified peptide bonds are taken up less efficiently (15–19). Thus, the unusual configurations of the cell wall peptides suggest that they are unlikely to be good substrates for the three known peptide permeases. We therefore set out to determine whether the cell wall peptides are taken up via one or more of the three general peptide permeases or whether an additional, cell wall peptide-specific transport system exists. Furthermore, if peptide transport mutants were found to be deficient in cell wall peptide recycling, this would provide an unambiguous demonstration that recycling involves the uptake of intact peptides rather than periplasmic hydrolysis followed by reutilization of the constituent amino acids.

Uptake of cell wall peptides by *S. typhimurium*. The uptake of cell wall peptides has previously been studied for *E. coli*. However, because the peptide transport systems are more completely characterized for *S. typhimurium*, it was important to establish that the same cell wall peptide recycling reaction also occurs in this species. The uptake of exogenously supplied cell wall peptides can be followed by measuring their incorporation into murein. Unlike peptides originating from proteins, cell wall peptides are not degraded to amino acids when they enter the cytoplasm. Instead, the intact peptide is attached directly to UDP–*N*-acetylmuramic acid and enters the pool of murein precursors. Thus, when radiolabeled cell wall peptides are added to a culture of *E. coli*, the label is incorporated only into murein and its precursors and not into proteins or other cellular polymers. Cell wall peptides were prepared from *E. coli* W7 (*dap lys*) as described previously (2). Briefly, isolated murein was digested with human murein amidase, and the tri- and tetrapeptide digestion products were purified by passing the sample over a Fractogel TSK-HW-40-S column (2.5 by 60 cm; E. Merck AG, Darmstadt, Federal Republic of Germany) (2, 20). The concentration of cell wall peptides was determined by measuring their A_2pm content after acid hydrolysis (23). To obtain tritiated cell wall peptides, cells were grown in the presence of diamino[3,4,5-^{3}H]pimelic acid (25 Ci mmol^{-1}; Commisariat à l'Energie Atomique, Paris, France).

To determine whether *S. typhimurium* incorporates ^{3}H-cell wall peptides specifically into murein, wild-type *S. typhimurium* LT2 was grown to an optical density at 578 nm

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TABLE 1. Bacterial strains

Strain	Genotype	Source or construction (reference)
S. typhimurium		
LT2(A)	Wild type[a]	B. N. Ames
CH44	LT2 Δ*oppBC250*	(8)
CH356	LT2 Δ*oppBC250 tppB16*::Tn*10*	(1a)
CH725	LT2 Δ*oppBC250 tppB16*::Tn*10 dpp-101*::Tn*5*	(6, 14)
CH1104	CH1279 *oppA324 tppB16*::Tn*10*	Donor P22 lysate, CH725; recipient, CH1432
CH1105	CH1279 *oppA324 dpp-101*::Tn*5*	Donor P22 lysate, CH725; recipient, CH1432
CH1110	CH1279 *oppA324 tppB16*::Tn*10 dpp-101*::Tn*5*	Donor P22 lysate, CH725; recipient, CH1104
CH1279	Wild type	M. D. Manson
CH1417	CH1279 *tppB16*::Tn*10*	Donor P22 lysate, CH725; recipient, CH1279
CH1421	CH1279 *tppB16*::Tn*10 dpp-101*::Tn*5*	Donor P22 lysate, CH725; recipient, CH1417
CH1432	CH1279 *oppA324*	*opp* derivative of CH1279
E. coli		
HB101	*hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	(7)
CH212	*hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44 oppA462*	Spontaneous *oppA* mutant of HB101 (7)
NO801	*thi lac pro galE*	(10)
CH483	*thi lac pro galE* Δ(*trp-tonB-oppABCDF*)*467*	*trp-opp* deletion selected in NO801
W7	*lysA dap*	(19)
CH1467	*lysA dap opp-468*	Spontaneous *opp* mutation of W7

[a] Two wild-type *S. typhimurium* strains were used as parents for the introduction of peptide transport mutations. CH1279 is a motile LT2 derivative and expresses *dpp* at about 10 times the level that LT2(A) does due to an uncharacterized mutation (14; unpublished results).

(OD_{578}) of 0.15 in minimal C medium (5) supplemented with glucose (0.2%), $MgSO_4$ (0.25 mg ml^{-1}), and each of the 20 protein amino acids except cysteine (13) at 20 μg ml^{-1}. This medium was used throughout this study. ^{3}H-cell wall peptides (1 nmol) were added to 1 ml of culture, and the cells were grown for a further 60 min at 37°C. As a control, the same strain was also grown in the presence of 1 nmol of [^{3}H]A_2pm. The cells were then collected by centrifugation, suspended in 1 ml of hot (100°C) sodium dodecyl sulfate solution (4%), and boiled for 5 min. Unlabeled carrier murein (0.1 mg) was added, and the cell walls were collected by centrifugation (10 min, 100,000 × *g*) and washed once with 1 ml of distilled water. The amount of radiolabel incorporated into cell wall material was determined by scintillation counting. To further identify the intermediates derived from the radiolabeled cell wall peptides, the spent medium and the sodium dodecyl sulfate-supernatant were separated on a Fractogel TSK-HW-40-S column (2, 21). The radioactivity in fractions corresponding to the bactoprenol-linked muropeptide precursors, UDP–*N*-acetylmuramylpeptides, free cell wall peptides, and A_2pm was then determined. When cells were incubated with radiolabeled cell wall peptides, label was recovered only in murein (46% of the total incorporated label), murein precursors (UDP–*N*-acetylmuramylpeptides and bactroprenol-linked muropeptides, 39%), and free wall peptides (13%); no significant amount of label was recovered in protein or in free A_2pm (<2%). The spent medium also contained no free [^{3}H]A_2pm. In contrast, when cells were incubated with free (non-peptide-linked) [^{3}H]A_2pm, 71% of the incorporated label was recovered in protein, while only 39% was found in cell wall material or its precursors. This demonstrates that like *E. coli*, *S. typhimurium* was able to reincorporate free cell wall peptides into murein. Furthermore, these peptides were not hydrolyzed to release free A_2pm during recycling.

Uptake of cell wall peptides by *S. typhimurium* peptide transport mutants. A series of *S. typhimurium* strains lacking one or more of the peptide permeases was constructed and is described in Table 1. The mutations harbored by these strains are well-characterized deletions or insertions which ensure complete inactivation of the relevant transport systems. Other than the transport lesions, these strains were isogenic. Each strain was tested for its ability to incorporate radiolabeled cell wall peptides into murein by using the methods described above (Table 2). Clearly, strains mutated for *opp* were unable to incorporate cell wall peptides into murein, while mutations in *tpp* and *dpp* had no effect on this recycling. Thus, only Opp was involved in the uptake of cell wall peptides.

Like other binding protein-dependent transport systems, Opp has many components and requires the function of a periplasmic peptide-binding protein (OppA) and a complex of membrane-associated proteins (8a–10). It was therefore important to address the possibility that not all of the Opp components are required for the uptake of cell wall peptides. For example, it is possible to envisage a scenario in which an alternative cell wall peptide-specific binding protein might interact with the Opp membrane-associated proteins. Cell wall peptide uptake was therefore examined in mutants specifically defective in individual components of Opp. A failure to take up cell wall peptides was observed for all *opp* mutants, including those which lacked only the periplasmic oligopeptide-binding protein (e.g., CH1432) and those which synthesize OppA but lack the membrane-associated components of the transport system (CH44). Thus, each of the Opp components was essential for cell wall peptide uptake.

To confirm that the failure of *opp* mutants to incorporate cell wall peptides into murein is due to their inability to take up these peptides, several of the mutants were assayed directly for the transport of ^{3}H-cell wall peptides. This was accomplished by a modification of the procedure described previously (6). Cells were grown in minimal C medium to an OD_{578} of 0.5, harvested by centrifugation, washed in phosphate buffer (20 mM, pH 7.2), and suspended in the same buffer containing 10 mM glucose. The cells (3 ml) were preincubated for 10 min at 37°C, after which time radiolabeled cell wall peptides were added. The mixture was incubated at 37°C, samples (0.5 ml) were removed every 30 s, and the cells were collected on a membrane filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.). The filters were

TABLE 2. Incorporation of cell wall peptides by *S. typhimurium* and *E. coli*

Strain	Relevant genotype	Transport phenotype	% Cell wall peptide incorporation into murein[a]
S. typhimurium			
LT2(A)	Wild type	Opp$^+$ Tpp$^+$ Dpp$^+$	20.3
CH44	LT2 Δ*oppBC250*	Opp$^-$ Tpp$^+$ Dpp$^+$	0.2
CH356	LT2 Δ*oppBC250 tppB16*::Tn*10*	Opp$^-$ Tpp$^-$ Dpp$^+$	0.4
CH725	LT2 Δ*oppBC250 tppB16*::Tn*10 dpp-101*::Tn*5*	Opp$^-$ Tpp$^-$ Dpp$^-$	0.1
CH1279	Wild type	Opp$^+$ Tpp$^+$ Dpp$^+$	29.5
CH1432	CH1279 *oppA324*	Opp$^-$ Tpp$^+$ Dpp$^+$	0.5
CH1104	CH1279 *oppA324 tppB16*::Tn*10*	Opp$^-$ Tpp$^-$ Dpp$^+$	1.2
CH1105	CH1279 *oppA324 dpp-101*::Tn*5*	Opp$^-$ Tpp$^+$ Dpp$^-$	0.7
CH1110	CH1279 *oppA324 tppB16*::Tn*10 dpp-101*::Tn*5*	Opp$^-$ Tpp$^-$ Dpp$^-$	0.8
CH1417	CH1279 *tppB16*::Tn*10*	Opp$^+$ Tpp$^-$ Dpp$^+$	16.5
CH1421	CH1279 *tppB16*::Tn*10 dpp-101*::Tn*5*	Opp$^+$ Tpp$^-$ Dpp$^-$	27.7
E. coli			
NO801		Opp$^+$ Tpp$^+$ Dpp$^+$	20.7
CH483	NO801 Δ*oppABCDF467*	Opp$^-$ Tpp$^+$ Dpp$^+$	0.7
HB101		Opp$^+$ Tpp$^+$ Dpp$^+$	17.3
CH212	HB101 *oppA462*	Opp$^-$ Tpp$^+$ Dpp$^+$	0.6

[a] ^{3}H-cell wall peptide (1 nmol) was added to 1-ml cultures of bacteria growing in minimal C medium (OD_{578}, 0.15). The cells were harvested after 30 min, and the murein was isolated. Data are given as the percentage of the total exogenous ^{3}H-cell wall peptide which was incorporated into murein per generation.

washed rapidly with four 5-ml portions of phosphate buffer prewarmed to 37°C, and the radioactivity retained by the filters was determined by scintillation counting. At a concentration of 1 μM, the rate of uptake of cell wall peptides by the wild-type strain (LT2) was 2 to 3 nmol min^{-1} mg of protein^{-1}, while strains CH44 (*oppBC*) and CH1432 (*oppA*) showed no measurable uptake (<0.01 nmol min^{-1} mg^{-1}).

Uptake of cell wall peptides by *E. coli* peptide transport mutants. For *E. coli*, Tpp and Dpp are far less well characterized than they are for *S. typhimurium*, and consequently, a comprehensive examination of the role of each system could not be carried out for this species. However, given that Opp seems to be the only system involved in the uptake of cell wall peptides by *S. typhimurium*, we examined the uptake of cell wall peptides in various *E. coli* strains carrying defined *opp* mutations (Table 2). For *E. coli*, cell wall peptide uptake was completely abolished in all *opp* mutants, including strains harboring a complete *opp* deletion (CH483) and a nonpolar *oppA* mutation (CH212). Thus, in *E. coli*, Opp also seems to provide the sole route for the uptake of cell wall peptides.

Affinity of Opp for cell wall peptides. The above results show that murein peptides were taken up by the oligopeptide permease but do not give any measure of the affinity of this permease for these peptides. In particular, it is interesting to determine the affinity for cell wall peptides relative to that for protein-derived peptides. To determine the relative affinity of Opp for cell wall peptides, we measured the abilities of several unlabeled peptides to compete with ^{3}H-cell wall peptides for uptake into the cell. To ensure that the peptides were only being transported by Opp, strain CH1421 (*tpp dpp*) was used. As expected, peptides known to be transported by Opp (17) inhibited the uptake of cell wall peptides (Fig. 1). Perhaps surprisingly, despite their unusual conformation, unlabeled cell wall peptides were more effective at inhibiting this uptake than were defined synthetic peptides. Assuming a single binding site for all peptides (22), the competition data in Fig. 1 can be used to calculate approximate K_m values for each of the peptides. For cell wall peptides, the K_m was 1.6 μM, typical of that for oligopeptides obtained by direct assay (18). However, the K_m for trilysine deduced from these competition experiments was 10-fold higher than that obtained previously by direct assay for transport (18). Similarly, the equivalent values for triserine and trialanine (70 and 144 μM, respectively) were 50- to 100-fold higher than those obtained by direct transport assay. Furthermore, none of the synthetic peptides tested was able to completely inhibit the uptake of ^{3}H-cell wall

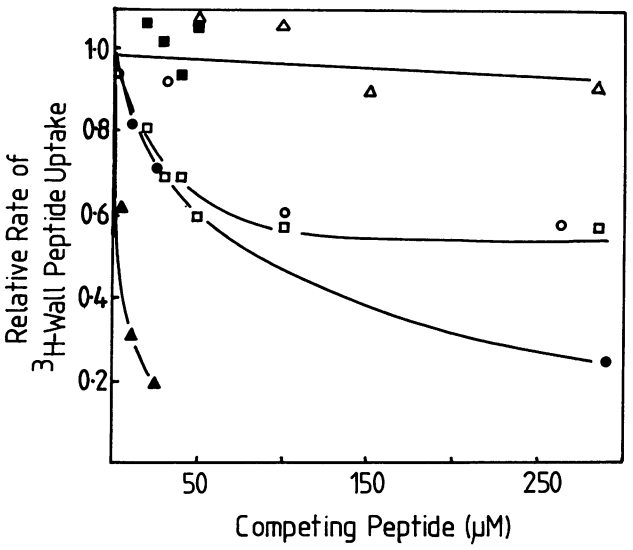

FIG. 1. Inhibition of ^{3}H-cell wall peptide uptake by unlabeled peptides. Cells of *S. typhimurium* CH1421 (Opp$^+$ Tpp$^-$ Dpp$^-$) were prepared as described in the text. ^{3}H-cell wall peptides were added to the cells at 1 μM together with unlabeled competing peptides at the indicated concentrations ranging from 0 to 300 μM. The rates of ^{3}H-cell wall peptide uptake in the presence of competing peptides are given relative to the rate obtained in the absence of competing peptides. Competing peptides: ▲, unlabeled cell wall peptides; ○, L-Ala-L-Ala-L-Ala; ●, L-Lys-L-Lys-L-Lys; ■, L-Leu-L-Leu-L-Leu; △, L-Glu-L-Glu-L-Glu; □, L-Ser-L-Ser-L-Ser. Concentrations of trileucine above 50 μM could not be used because of its limited solubility.

peptides, even when present in 300-fold excess. In addition, certain peptides known to be substrates for Opp, for example, trileucine and triglutamic acid (1a, 4, 6; unpublished results), had no detectable inhibitory effect on the uptake of cell wall peptides. Taken together, these apparent discrepancies can be explained if it is assumed that OppA has more than one peptide-binding site, each with different but overlapping specificities. This possibility has previously been suggested by Guyer et al. (4) on the basis of substrate-binding competition experiments with the purified OppA protein. Further support for this idea comes from our isolation of *oppA* mutants which are totally resistant to the toxic peptides triornithine and trilysine but are unaffected in the utilization of prolylglycylglycine (8). As OppA must be able to recognize 8,000 different protein-derived tripeptides, as well as the cell wall peptides, the evolution of multiple binding sites with overlapping specificities is clearly a means of achieving a broader specificity. OppA is the largest known periplasmic binding protein (M_r = 59,000) which may reflect this function. Inspection of the sequence of the OppA protein (9) does not show an obvious duplication of any domain within the protein.

Physiological role for cell wall peptide recycling via Opp. Since most cell wall peptides which are released from murein are taken up into the cytoplasm and reused to make new murein, these peptides would be expected to be an important source of murein precursors. Thus, Opp$^-$ strains might be expected to show specific growth defects. To estimate the importance of recycling, an *E. coli dap* mutant (strain W7) and an otherwise isogenic *opp* derivative (CH1467) were grown in minimal C medium with various concentrations of A_2pm. Once the cells utilize all the A_2pm in the medium, they lyse unless they are able to reutilize those cell wall peptides previously released from the murein. The *opp* derivative lysed sooner than its *opp*$^+$ parent did when grown with limiting A_2pm (1.0 or 1.5 μg ml^{-1}), and this strain required more A_2pm to reach maximum turbidity (Fig. 2). Furthermore, cell wall peptides could substitute for the A_2pm requirement of the Opp$^+$ strain (W7) but not for that of the Opp$^-$ strain. Thus, the ability to recycle cell wall peptides clearly serves a physiologically important role.

The experiments described above demonstrate that the oligopeptide permease was required for the uptake of cell wall peptides. In *opp* mutants, no uptake of cell wall peptides could be detected, while in contrast, mutants lacking both Dpp and Tpp transported cell wall peptides normally. It is therefore apparent that Dpp and Tpp play no role in cell wall peptide uptake and, in addition, that there is no cell wall peptide-specific permease. The finding that Dpp and Tpp were not involved in the uptake of cell wall peptides is not, perhaps, surprising. Cell wall peptides are hydrophilic tri- or tetrapeptides and would not seem to be ideal substrates for these systems. More surprising is the finding that these peptides were good substrates for Opp, which has generally been considered to have poor affinity for peptides containing D-amino acids or γ-peptide bonds. Presumably, cell wall peptides provide a special case, as they consist of a few, defined sequences and are certainly the most frequently encountered peptides. As such, they may have provided a strong selection for the evolution of Opp specificity.

The oligopeptide permease genes are expressed constitutively (11). This is somewhat unusual as most transport systems are tightly regulated and are only expressed when substrate is present. The finding that Opp was required for the recycling of cell wall peptides provides an explanation for this observation, as cell wall peptide substrates are present even when peptides are not added exogenously. The role of Opp in the uptake of cell wall peptides also provides an explanation for the finding that the dipeptide-binding protein, and not the oligopeptide-binding protein, serves as a chemotactic receptor for peptides (14). Were OppA to function as a chemoreceptor, the ability to bind and transport cell wall peptides, which are ever-present in the periplasm, would not assist gradient sensing.

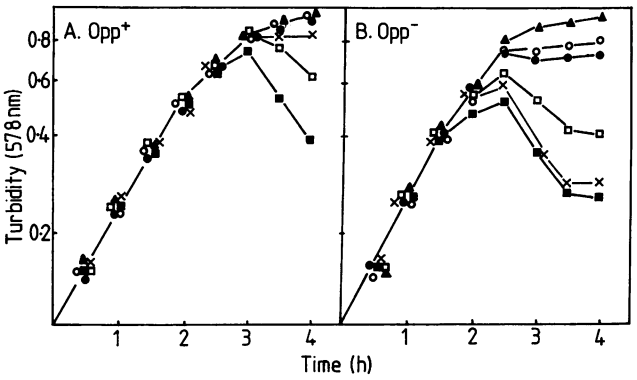

FIG. 2. Effect of limiting A_2pm on the growth of Opp$^+$ and Opp$^-$ *E. coli*. Cells of strains W7 (Opp$^+$) and CH1467 (Opp$^-$) were grown to an OD_{578} of 0.6 in minimal C medium containing 10 mg of A_2pm ml^{-1}. The cells were collected by centrifugation, washed, and suspended to an OD_{578} of 0.1 in the same medium lacking A_2pm. A_2pm was added to the following concentrations (micrograms milliliter^{-1}): 1 (■); 1.5 (□); 2.0 (●); 3.0 (○); and 4.0 (▲). In addition, growth in 1.0 μg of A_2pm ml^{-1} was monitored with 10 μg of cell wall peptides ml^{-1} (×) added. Growth was followed by measuring the OD_{578}.

The results presented here provide unambiguous evidence that cell wall peptides are recycled during all growth and that this involves the intact uptake of peptides into the cytoplasm prior to reincorporation into new cell wall material. It has been estimated that as much as 50% of the cell wall peptides may be recycled per generation. We have demonstrated here that, at least under certain conditions, this process is physiologically important. The availability of mutants defective in the recycling of cell wall material will now enable us to fully assess the role of murein turnover during bacterial cell growth.

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