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Hydrophobic Peptide Auxotrophy in *Salmonella typhimurium*

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The growth of a pleiotropic membrane mutant of *Salmonella typhimurium* with modified lipopolysaccharide composition was found to be strictly dependent on the peptone component of complex media. Nutritional shiftdown into minimal media allowed growth for three to four generations. Of 20 commercial peptones, only enzymatic digests supported growth to varying degrees. Neither trace cations, amino acids, vitamins, carbohydrates, lipids, glutathione, polyamines, carbodiimides, nor synthetic peptides stimulated growth; however, cells still metabolized carbohydrates, and amino acid transport systems were shown to be functional. A tryptic digest of casein was fractionated into four electrophoretically different peptide fractions of 1,000 to 1,200 molecular weight which supported growth to varying degrees. The best of these was further fractionated to two highly hydrophobic peptides. N-terminal modifications eliminated biological activity. Fluorescein-conjugated goat antibody to rabbit immunoglobulin G was used as a probe to detect antipeptide antibody-peptide complexes on membrane preparations. Cells grown on peptone distributed the peptide into both inner and outer membranes. The peptide could be removed with chaotropic agents, and cells had to be pregrown in peptone-containing media to bind the hydrophobic peptide. The gene (*hyp*) responsible for peptide auxotrophy was mapped at 44 to 45 units by conjugation.

The role of peptides in the nutrition of microorganisms is poorly understood. Before utilization as a carbon, nitrogen, sulfur, or amino acid source, peptides must be hydrolyzed to amino acids either before or after absorption. The growth response to a particular peptide depends on at least two factors: transport and cleavage of the peptide or amino acids, depending on which event occurred first. Bacteria are known to possess peptide uptake systems which are distinct from those for amino acids, and it appears that a multiplicity of oligopeptide transport systems exist in gram-negative organisms (18, 25). Surprisingly, the utilization of amino acids derived from peptides is more effective than that derived from the corresponding amino acids (25). No satisfactory explanation has been offered for the nutritional superiority of peptides over equivalent amino acid mixtures, although it has been speculated that peptides are more active because they hydrolyze at a rate which is adequate for protein synthesis without producing high amino acid levels susceptible to degradation (16). We are aware of no evidence that peptides per se can be incorporated into proteins, and there is no known specific peptide effect on bacterial growth. It has been suggested, however, that peptides may covertly facilitate the transport of other substances by "illicit transport" (1). The only organism reported to date which has a natural absolute requirement for oligopeptides is *Thermoplasma acidophilum*, although the role of this peptide(s) is still uncertain (31).

In this paper we report the first description of an absolute peptide requirement for growth in a mutant of *Salmonella typhimurium*. This mutant has been studied intensively by us and can be best described as an outer membrane (OM) mutant with pleiotropic phenotypes (L. V. Brãnes, Ph.D. thesis, University of Saskatchewan, Saskatoon, Canada, 1980). The membrane composition of this strain is similar to that of the parent with respect to fatty acid, phospholipid, and protein but differs significantly in the composition of its lipopolysaccharide (LPS) (L. V. Brãnes and W. W. Kay, manuscript in preparation). The most striking phenotype of this mutant is its absolute growth dependence on peptone, the biologically active component of which is an unusually hydrophobic peptide which readily adsorbed to the bacterial membrane. In this paper we describe the resolution

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of the peptone auxotrophy to the requirement for a hydrophobic peptide and show its localization in the cell envelope. To our knowledge the existence of peptide auxotrophy in the absence of specific amino acid auxotrophy has never been observed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. typhimurium* strain SA33 (*proA26 tre*$^+$ *clb*$^+$ *galE446 rfa-630* F$^-$) is a "deep rough" mutant; it was obtained from K. E. Sanderson. Strain KS643 is a spontaneous mutant initially isolated as resistant to saturating concentrations of dicyclohexylcarbodiimide on nutrient agar under anaerobic conditions. It was further found to be resistant to a wide range of inhibitors and has been characterized as a strain producing an abnormal LPS. The details of the isolation and characterization will be reported elsewhere (L. V. Brānes and W. W. Kay, manuscript in preparation). Cells were routinely grown by shaking in a gyratory shaker at 37°C in Davis minimal medium supplemented with nutrient broth. Cells were usually harvested by centrifugation at 10,000 × *g* and washed a minimum of three times with carbon-free minimal medium. For nutritional experiments, washed cells were inoculated (0.1%) into minimal medium containing 10 mM glucose and 50 μg or proline ml^{-1}. Growth factors were added at various concentrations. Incubations were carried out at 37°C, and growth was measured either with a Klett-Summerson colorimeter equipped with a no. 66 filter and previously calibrated to cell dry weight or spectrophotometrically at 650 nm.

Fractionation of the cell envelope. The separation of OM from cytoplasmic membrane (inner membrane [IM]) was achieved by sucrose density centrifugation. A better resolution was obtained with strain KS643, using a slight modification of the gradient. Due to the higher density of the OM in this mutant, a larger sucrose cushion was required in these gradients. Good separation in the case of SA33 was obtained by using a gradient of 1.5 ml of 2.02 M sucrose, 5.6 ml of 1.44 M sucrose, and 5 ml of 0.77 M sucrose. The modification was required due to the differences in density of the OM fractions of the various strains. In both strains two distinct bands were visible and widely separated, the upper band representing IM and the lower band representing OM. No formation of M band (unseparate IM from OM) was observed in either case; the IM was free of OM contamination as judged by the absence of 2-keto-3-deoxyoctulosonic acid, and the OM contained less than 0.5% of the IM enzymes succinic dehydrogenase (EC 1.3.99.1) and NADH oxidase (EC 1.6.99.3). Membranes were prepared and separated by modifications to the method of Koplow and Goldfine (13).

Transport assays. Amino acid transport assays were performed by membrane filtration, using 0.35 mg (dry weight) of washed cells in carbon-free minimal medium. Labeled amino acids were added to 50 μM concentrations.

Isolation of peptide fraction II. A saturated solution of an enzymatic digest of casein (Fisher peptone no. 3) was fractionated by Sephadex G-10 gel filtration. The column was washed with 0.5% NaN_3 before use. The inhibitor was removed, and column fractions were developed with sterile distilled water. A 5-ml portion of boiled peptone (40%, wt/vol) was applied to a column (2.5 by 10 cm) and eluted in the cold with distilled water at a flow rate of 0.5 ml min^{-1}. Ten fractionations were conducted, and the peak causing growth stimulation with KS643 was lyophilized. This crude fraction was further separated by preparative high-voltage electrophoresis at acid pH, and the nonmigrating fraction (neutral fraction II), detected by ninhydrin staining on a test strip, was eluted and lyophilized. This fraction was found to contain about 10 peptides by thin-layer chromatography. The composition was analyzed further by two-dimensional electrophoresis at pH 7.0, followed by chromatography in butanol-acetic acid-water (12:3:5, vol/vol). This analysis revealed 12 ninhydrin-positive spots, 8 of which were minor. For expediency, further purification was restricted to electrophoresis at pH 9.0, which resulted in fractionation of neutral fraction II into four electrophoretically distinct fractions (P1 to P4); these were then eluted and lyophilized. Neutral fraction P2 was used for immunological studies.

Antiserum preparation. Male rabbits (2 to 3 kg) were used for the immunization. Eight intradermal injections of a saline solution of peptide II were given to each animal. Every 5 days they received a further dose of 400 μg of antigen. Rabbits were finally exsanguinated by cardiac puncture 5 days after the last injection. All sera were stored at −20°C. As a control, all animals were bled before immunization.

Passive hemagglutination. Erythrocytes covalently linked to P2 by chromic chloride coupling (7) were used to evaluate the agglutinating activity of anti-P2 antisera. The peptide antigen (0.1 mg in saline) was added to chromic chloride (0.2 ml of 0.1% $CrCl_2$ in 0.9% NaCl), and an equal volume of thrice-washed, packed mouse erythrocytes was added immediately. After 10 min at room temperature, the mouse erythrocytes were washed three times with 10 ml of phosphate-buffered saline (PBS) and were suspended to a concentration of 1%. Decomplemented (56°C for 30 min) rabbit antiserum was serially diluted on microtiter plates and incubated with an equal volume of peptide-coated mouse erythrocytes (2% suspension) at room temperature for 10 to 16 h, after which the plates were examined for hemagglutination. Two different controls were done: one with prebled rabbit antiserum, and a second control with 50 mM PBS (pH 7.2) instead of rabbit anti-P2 antisera.

Immunofluorescence. The indirect "sandwich technique" (12) was used on membrane particles (cell envelope, IM, and OM). A membrane suspension (100 μl of 1 mg of protein per ml) was air dried, fixed with acetone for 10 min, and then washed three times in PBS (pH 7.2). Rabbit anti-P2 antiserum (0.2 ml) was added, incubated for 1 h in a humidified chamber at 37°C, and then washed four times in PBS. The fluorescein isothiocyanate-conjugated goat antibody (0.2 ml) to rabbit immunoglobulin G (anti-P2) was added (dilution, 1:5) and incubated for 1 h in a humidified chamber at 37°C. The following controls were done: saline, fetal calf serum, and prebled rabbit antiserum instead of rabbit anti-P2 antiserum. A control for

nonspecific binding was done, using fluorescein isothiocyanate-conjugated rabbit antibody to goat immunoglobulin G. All controls were incubated under conditions identical to those described above. After three consecutive washings in PBS, the membranes were mounted in glycerol-PBS (1:1), covered with a cover slip, and sealed with nail polish. The samples were examined under incident UV light in a Zeiss photomicroscope II, using a ×100 oil immersion lens, an HBO 200-W 3L2 lamp, and a BG12 filter for excitation, plus a no. 47 barrier filter. The observations were done by reflected fluorescence. Fresh specimens, i.e., those which had been neither frozen nor stored longer than overnight on ice, were used for photography.

Phage transductions and bacterial conjugations. Both P22 *int3* (smooth specific) Es18 and Es18h (rough and smooth specific; 14) phage were grown on strain LT2 or KS643 in Luria broth overnight. Phage titers were determined in a soft-agar overlay on Luria agar and routinely were about 10^{11} plaque-forming units ml^{-1}. Cell-free supernatants were stored at 4°C over $CHCl_3$. Transductions were performed at multiplicities of infection of 0.1 to 1.0, and transductants were scored at 20 h. For conjugations the various Hfr's used were first selected for active clones by replicating on a suitable F^- lawn, with a selection for an auxotrophic marker.

Chemicals. ^{14}C- and ^{3}H-labeled amino acids were obtained from Amersham Corp.; dicyclohexylcarbodiimide was from Aldrich Chemical Co.; ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate were from Pierce Chemical Co.; and histone type II, spermine, spermidine, trypsin inhibitor, tripeptides, and peptide derivatives were from Sigma Chemical Co. Fuorescein isothiocyanate-conjugated goat antibody and fluorescein isothiocyanate-conjugated rabbit antibody were from Miles-Yeda (lots S403 and S337, respectively). All other chemicals were analytical-grade reagents of commercial origin.

RESULTS

Peptone auxotrophy. Unlike its immediate ancestor SA33, KS643 was found to be unable to grow on glucose minimal medium supplemented with proline. In Table 1 are listed the wide range of supplements tested as potential growth requirements. Strain KS643 did not respond to any of the 18 different carbon sources known to be utilized by *S. typhimurium* or to a mixture of several of them. The combinations of amino acids, vitamins, purines, and pyrimidines used in Holliday pools (9) were also ineffective. Since this strain was originally isolated as resistant to dicyclohexylcarbodiimide (Brãnes, Ph.D. thesis) and since carbodiimides have been shown to restore membrane function in some organisms, we also tested the three carbodiimides to no avail. A lipid requirement was also eliminated since the chloroform-methanol extracts were without effect, as were various polyamines. Since the mutant has an unnatural LPS, we also tried

TABLE 1. *Strict peptone growth requirement of S. typhimurium KS643*

Medium supplement	Growth[a]	
	SA33	KS643
Any of 18 carbon sources (0.5%)	+	−
Glucose + L-malate + citrate (0.5%)	+	−
Holliday pools[b]	+	−
Carbodiimides[c]	+	−
$CHCl_3$-CH_3OH extract[d] of nutrient broth (0.5 mg ml^{-1})	+	−
Nutrient broth residue[d] (2 mg ml^{-1})	+	+
Polyamines (spermine, putrescine; 2 mg/ml)	+	−
Cations (Co, Ca, Mn, Mo; 0.1 mM)	+	−
LPS (1 mg ml^{-1})	+	−
Bovine serum albumin (2 mg ml^{-1})	+	−
Peptone (1%)	+	+
Acid-hydrolyzed peptone[e]	+	−
Peptone + EDTA[f]	+	+
Casamino Acids (0.1%) + Trp + Cys (50 μg ml^{-1})	+	−
Trypsine inhibitor (2 mg ml^{-1})	+	−
L-Leucyl-L-leucyl-L-leucine (2 mg ml^{-1})	+	−
L-valyl-L-valyl-L-valine (2 mg ml^{-1})	+	−

[a] Growth tests were done in glucose minimal medium plus proline (50 μg ml^{-1}).

[b] Amino acids at 50 μg ml^{-1}; vitamins at 5 μg ml^{-1} (9).

[c] Carbodiimides: *N,N'*-dicyclohexylcarbodiimide (DCCD), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCD), and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide. All were tested at 10^{-3} and 10^{-4} M. Only DCCD was toxic at 10^{-3} M to SA33.

[d] A 10-ml portion of nutrient broth was extracted four times, and the dried residue was made up in minimal medium.

[e] Hydrolyzed at 100°C in 6 N HCl for 20 h.

[f] Peptone was treated with 0.2 M EDTA, and the EDTA was removed by electrophoresis.

LPS isolated from a smooth LT2 strain; however, it was also ineffective. A mixture of divalent cations at relatively low concentrations was also without effect, and the minimal medium already contained relatively high concentrations of K^+, Na^+, Fe^{2+}, and Fe^{3+}. Only whole peptone restored growth, in contrast to acid-hydrolyzed peptone, Casamino Acids plus tryptophan and cysteine, trypsin inhibitor, or the two tripeptides shown. We therefore considered that peptides present in peptone constituted the growth requirement for KS643.

Growth response to peptone. When carbon-free minimal medium plus proline was supplemented with increasing peptone, the growth response of KS643 was linear up to 2% peptone, indicating that the cells could use peptone as a carbon source. No growth rate stimulation was observed by glucose in glucose minimal medium supplemented with various quantities of peptone (0.01 to 2%); however, higher growth yields were achieved than with peptone alone (data not shown). Also, in peptone-supplemented N-free glucose minimal medium [Davis minimal medium in which $(NH_4)_2SO_4$ had been replaced by

K_2SO_4], KS643 cells grew normally, suggesting that N metabolism was also functional. When washed cells were shifted down into glucose minimal medium (Fig. 1), they underwent three to four divisions at about the same rate as parent strain SA33 before ceasing growth. At the same time, only a gradual doubling in optical density occurred up to 6 h, after which no further increase was measured up to 24 h. This suggests that the cells contained only enough peptide for limited growth and became smaller upon division. Thus, the mechanisms of cell division were apparently intact.

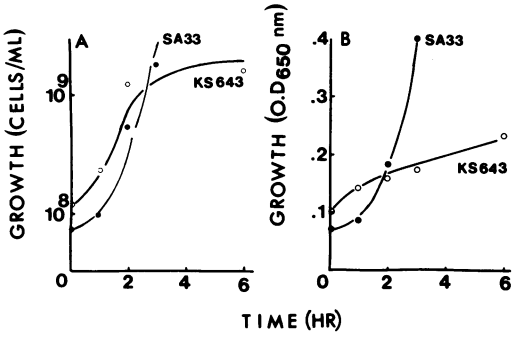

FIG. 1. *Effect of peptide starvation of S. typhimurium SA33 and KS643. Cells were grown on complex medium, washed several times, and suspended in glucose minimal salts plus proline. Growth was followed (A) by direct viable counts and (B) by turbidity. OD, Optical density.*

Growth response to commercial peptone. From an examination of the growth yields obtained from 20 commercial peptones (Table 2), two important generalities concerning the peptide requirement were found: all peptones regardless of source were effective, but to widely varying degrees; and peptones prepared by acid hydrolysis were poor compared with enzymatic digests whether or not they were resupplemented with tryptophan and cysteine. This indicated that it was unlikely that a particular sequence-specific peptide was required but that a peptide of a particular size or of tryptophan or cysteine content was likely to be required since acid hydrolysis usually produces a greater degree of fragmentation and destroys these two residues. Four of these commercial peptones were further fractionated by gel filtration into several sizes of peptides, and the growth response of KS643 was assayed on all of the fractions (Fig. 2). Figure 3A and B represents two different enzymatic digests of casein. In each case the major growth occurred in response to a single size class of peptide. From a comparison with several peptide standards on calibrated G-10 and G-25 columns, we estimated this size to be 1 to 1.2 kilodaltons. A rather varied growth response to the complex protein pancreatic digest was shown (Fig. 3C), indicating that casein contains

TABLE 2. *Growth response of S. typhimurium KS643 to commercial peptones*

Peptone (2%)	Source[a]	Treatment	Protein	Growth[b] (mg [dry wt] ml^{-1})
Pepticase	HS	Tryptic	Casein	0.22
Hy-Case S.F.	HS	Acid (partial)	Casein	0.05
N-Z-Amine AS	HS	Pancreatic	Casein	0.44
N-Z-Case M	HS	Tryptic	Casein	0.07
Edamin S	HS	Tryptic	Lactalbumin	0.15
Hy-Case-Amino	HS	Acid	Casein	0.02
Proteose no. 3	Difco	Pancreatic	Meat	0.74
Casein hydrolysate	GIBCO	Pancreatic	Casein	0.35
Acidicase	BBL	Acid	Casein	0.01
Soy peptone no. 110	GIBCO	Papaic	Soybeans	0.39
Lactalysate	BBL	Pancreatic	Lactalbumin	0.24
Casein hydrolysate	NBC	Acid	Casein	0.02
Casamino Acids	NBC	Acid	Casein	0.0
Peptone	Difco	Enzymatic	Meat	0.34
Gelatin hydrolysate	GIBCO	Pancreatic	Gelatin	0.13
Meat hydrolysate	GIBCO	Pancreatic	Meat	0.33
Meat casein	GIBCO		Casein	0.19
Tryptic soy	Difco	Tryptic	Soybean	0.32
Peptone no. 3	Fisher	Tryptic	Casein	0.21
Trypticase peptone	BBL	Pancreatic	Casein	0.43

[a] HS, Humko-Sheffield Chemical, Lyndhurst, N.J.; Difco Laboratories, Detroit, Mich.; GIBCO Diagnostics, Madison, Wis.; BBL Microbiology Systems, Cockeysville, Md.; NBC, Nutritional Biochemicals Corp., Cleveland, Ohio; Fisher Scientific Co., Pittsburgh, Pa.

[b] Growth was measured spectrophotometrically at 650 nm. Cells were grown in 10 mM glucose–50 μg of proline per ml (minimal medium) with 2% peptone supplements.

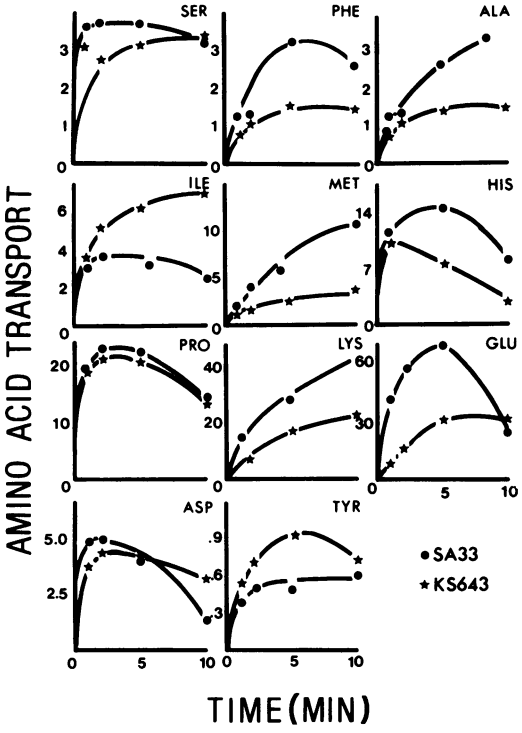

FIG. 2. *Amino acid transport in S. typhimurium SA33 and KS643. Incorporation into whole cells was measured by filtration, and the units shown are nanomoles per milligram (dry weight) of cells.*

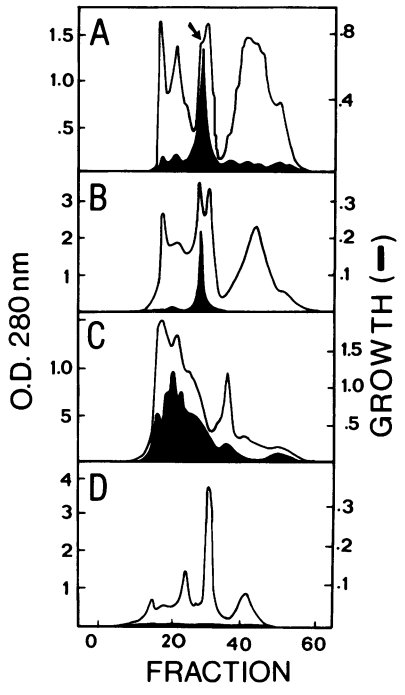

FIG. 3. *Sephadex G-10 fractionation of commercial peptones. The solid lines represent optical density at 280 nm (O.D. 280 nm), and darkened areas represent the growth response of KS643 to the various fractions as measured by turbidity at 650 nm. The peptones used were: (A) Fisher peptone no. 3; (B) N-Z-amine AS; (C) Difco proteose peptone no. 3; (D) Acidicase (Table 2).*

a size class of peptide which stimulates growth, but that the size requirement is not necessarily absolute. A poor casein peptone (Table 2) was also fractionated in this way (Fig. 3D), which confirmed that the majority of the peptides were of low molecular weight and did not provide the appropriate peptide for growth of KS643.

Effect of peptide modifications on growth of KS643. The growth-promoting peak illustrated in Fig. 3A was used to assess the effect of peptide modifications on growth stimulation (Table 3). Caseins are rich in phosphate as well as in carbohydrate (galactose, galactosamine, *N*-acetylneuraminic acid), which may have provided the growth requirements. However, neither extensive digestion with alkaline phosphatase, glucose oxidase, and galactose oxidase nor periodate oxidation had any effect on the biological activity of this peptide fraction. Also, the addition of these carbohydrates, either singly or in combinations, was ineffective. N-terminal modifications by either dinitrophenylation or acetylation precluded growth. This also confirmed that the active fraction was a peptide.

Amino acid transport. Oligopeptide and dipeptide transport systems have been elucidated with various organisms, using amino acid auxotrophs, the peptide supplying the required amino acid. In the unlikely event that KS643 was auxotrophic for one or more amino acids and that the transport systems for these were largely defective, we measured the uptake rates of amino acids representing these systems (Fig. 2). Although differences were observed, they were not considered significant. L-Glutamate transport, however, was only about 25% of that of the parent strain. In contrast, L-glutamine transport (data not shown) was higher in KS643.

Peptide fractionation and composition. In an attempt to determine whether casein contained a specific peptide required by KS643, we further fractionated the pooled active fraction (Fig. 2A). A summary of the fractionation procedures is presented in Table 4. The result of these purifications produced a highly enriched fraction (P2) which still contained three peptides, two of which were minor. Surprisingly, none of the 12 peptides contained in FII were found to contain His, Arg, Trp, or Tyr as detected by amino acid-specific sprays. The four

most purified, electrophoretically distinct fractions (P1 to 4) were all found to support growth to varying degrees (Table 5); the most active was P2. This again underlines the lack of sequence specificity required to support growth.

TABLE 3. *Peptide modifications and growth response of S. typhimurium KS643*

Treatment[a]	Condition	Growth (mg [dry wt] ml^{-1})
None		0.19
Alkaline phosphatase	5 U, 20 h, RT	0.18
Glucose oxidase	8 U, 20 h, RT	0.19
Galactose oxidase	35 U, 20 h, RT	0.20
Neuraminidase	5 U, 20 h, RT	0.20
Periodate[b]		0.18
Dinitrophenylation[c]		0
Acetylation[d]		0

[a] One milligram of peak III (Fig. 3A) was treated (sterile), heat inactivated by boiling for 5 min, and added to growth media as in Table 6. RT, Room temperature.

[b] Reacted with 0.04 M $NaIO_4$ in 0.05 M sodium acetate for 24 h at 4°C. Reaction was terminated with excess ethylene glycol, and the peptide was separated on Sephadex G-10, dried, and used for growth studies.

[c] Reaction with excess dinitrofluorobenzene at pH 8 in (0.1 M) $NaHCO_3$. Excess reagent was ether extracted, and the dried residue was used for growth studies.

[d] Reaction with excess acetic anhydride at 0°C for 30 min and then for 3 h at RT. Reagent was destroyed with HCl and neutralized and sterilized for growth studies.

The yield of P2 from peptone was estimated to be approximately 1%.

Fractions FII and P2 were subjected to amino acid analysis, and the results confirmed the absence of Lys and Arg and the near absence of Tyr (Table 6). The striking feature of both fractions was their highly hydrophobic nature, the absence (P2) or near absence (FII) of aromatic residues, and the peculiarly high leucine content.

Location of P2 on the bacterial membrane. Since the major defect associated with strain KS643 is an alteration in LPS composition, we have assumed that the inability to grow in the absence of peptides concerns some modification of the bacterial membrane. The demonstration of specific binding or localization in the membrane of these peptides proved to be exceedingly difficult. Radiolabeling peptide 2 with ^{125}I, using chloramine-T or Iodogen, proved impossible since no Tyr or His residues were present. Also, reductive methylation with ^{14}C-labeled acetic anhydride could not be used due to the lack of Lys residues and the growth sensitivity to N-terminal blocking. As a result, an immunological approach was undertaken. Anti-P2 antisera was raised in rabbits. It is difficult to detect the presence of non-precipitating antibody to peptides, so erythrocytes to which the

TABLE 4. *Summary of peptide fractionation procedures*

Separation method	Analysis	Result(s)
Gel filtration	Sephadex G-10	Single peak (mol wt, ~1,000–1,500) isolated (FI)
PHVPE[a] on FI	HAc/Pyr/H_2O[b]	Four peptide fractions isolated; neutral most active (FII)
TLC[c] on FII	Solvent[d] 1	Seven peptides
	Solvent 2	Eight peptides
	Solvent 3	Ten peptides
	Solvent 4	Ten peptides
2-D fingerprint FII	Electrophoresis[e] and chromatography[f]	Twelve peptides
	Sprays[g]	
	Pauly	No histidine
	Sakaguchi	No arginine
	Ehrlich	No tryptophan
	Naphthol	No tyrosine
PHVPE[e] on FII		Four peptide fractions (P1–4); P2 most active

[a] PHVPE, Preparative high-voltage paper electrophoresis, 1,200 V for 75 min.

[b] Acetic acid-pyridine-water (10:1:89, vol/vol).

[c] TLC, Thin-layer chromatography on cellulose.

[d] 1, Butanol-acetic acid-water (12:3:5, vol/vol); 2, butanol-acetic acid-water (4:1:5, vol/vol); 3, butanol-acetic acid-pyridine-water (150:30:100:120, vol/vol); 4, pyridine-isoamyl alcohol-water (6:6:7, vol/vol).

[e] Ammonium carbonate (0.1 M, pH 9), 1,500 V for 60 min.

[f] Butanol-acetic-water (12:3:5, vol/vol).

[g] Pauly, Sulfanilic acid; Sakaguchi, 8-hydroxyquinoline; Ehrlich, *p*-dimethylaminobenzaldehyde; naphthol, α-nitroso-β-naphthol, 0.1% (wt/vol) in 96% ethanol.

TABLE 5. *Growth response of S. typhimurium KS643 to electrophoresis fractions*

Fraction[a]	Growth (mg [dry wt] ml^{-1})
P1	0.15
P2	0.24
P3	0.08
P4	0.09

[a] Fractions were added sterile to minimal medium at 1 mg ml^{-1}.

TABLE 6. *Amino acid analysis of peptide fractions*[a]

Amino acid	Mol%	
	Fraction 2	Peptide 2
Asp	4.20	—[b]
Thr	6.70	7.40
Ser	8.00	8.80
Glu	1.50	
Pro	6.70	7.40
Gly	3.00	1.90
Ala	9.10	10.00
Val	12.50	13.70
Met	6.30	6.90
Ile	10.90	12.00
Leu	29.00	31.09
Tyr	0.99	—
Phe	0.94	—
His	0.13	—
Lys	—	—
Arg	—	—
% Hydrophobic	60.63	64.05

[a] Analyses were done on a Beckman amino acid analyzer, model 118BL. Samples were hydrolyzed in 6 N HCl at 100°C for 24 h in vacuo.

[b] —, Not detectable.

peptide antigen (P2) was coupled with chromic chloride was used in a passive hemagglutination assay. Fluorescein-conjugated goat anti-rabbit immunoglobulin G antibody was used as the immunological probe to detect the presence of cell membrane-P2-anti-P2 complexes. Inner and outer cell membranes from strains SA33 and KS643 were separated by sucrose density gradient centrifugation, and the presence of the peptide (P2) was detected by reflected fluorescence microscopy. When cells were grown in peptide-containing media, the peptide was detected in both IM and OM preparations of SA33 and KS643 (Fig. 4A and B). Washing whole membrane preparations (IM and OM) five times in 50 mM phosphate buffer containing one of the following chaotrophs, 3 M KCl, 5 M urea, 5 M guanidine hydrochloride, or 5 M guanidine thiocyanate, rendered the membrane completely negative toward immunological detection of the

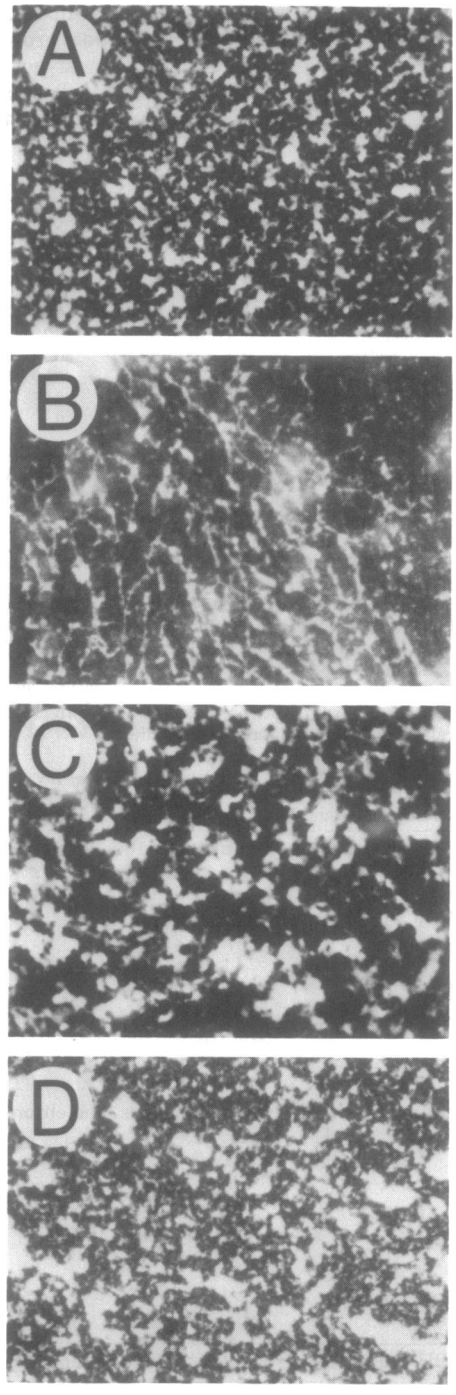

FIG. 4. *Immunofluorescence of membranes from S. typhimurium. Membranes were treated with fluorescein-conjugated anti-rabbit immunoglobulin G after incubation with anti-P2 rabbit antiserum. Membrane fractions are indicated as follows: SA33—(A) IM, (B) OM; KS643—(C) IM, (D) OM.*

peptide (P2). Fluorescence was consistently absent in controls for autofluorescence as well as in those for nonspecific binding. The peptide was therefore considered not to be covalently bonded to membrane components, unless these components were also removed by these chaotrophs. Interestingly, when cell membranes of SA33 cells grown in glucose minimal medium were incubated either in nutrient broth or in the presence of peptide (P2) at 37°C for 30 min, no peptide could be detected adsorbed to the membrane after washing with PBS. The bacteria first had to be grown in the growth factor to be able to effectively incorporate it into the membrane (Fig. 4B).

Genetic analysis of peptide auxotrophy. Initially, unusual difficulty was encountered in locating the position of the mutation in strain KS643 because the strain is resistant to many antibiotics, including streptomycin commonly used in counterselection procedures, and also because of the unusual phage resistance, especially to phages P22 and P1 commonly used in transductions (30). However, transducing phages Es18 and Es18h1, previously used in mapping experiments with OM-defective mutants (4), were found to infect both the LT2 wild-type strain and KS643, but not SA33. Phages were grown on the wild-type strain LT2, and several attempts were made to transduce KS643 from peptide auxotrophy to prototrophy, all without success. Control experiments indicated that these phage preparations did in fact transduce other genetic markers in other strains. From these results it was assumed either that KS643 carried multiple mutations resulting in the peptide auxotrophy or that the SA33 genetic background, i.e., the *rfa* gene, was required for expression of this phenotype. Bacterial conjugation was therefore attempted as a means of locating the gene for peptide auxotrophy. Fertile clones of 14 stock Hfr's were isolated, two of which appeared to readily correct the *hyp* (hydrophobic peptide auxotrophy) gene (Hfr's 654 and 535 [Fig. 5]) and appeared to enter close to the origin of Hfr 535. In timed interrupted mating experiments, every 2.5 min, the entry of hyp^+ occurred within 4 min of mating, which is equivalent to 70 min on the old *S. typhimurium* map (13) and to 49 units on the most recent map (14). Since the points of entry of seven of the Hfr's used are in the region of the *rfa* gene, it is unlikely that the regain of *rfa* alone could suppress the Hyp phenotype.

KS643 could also be spontaneously reverted by selection in liquid minimal medium. The revertants all grew similarly to the parent SA33 strain on minimal medium and maintained the original proline auxotrophic marker. From these experiments it was concluded that there is likely a single genetic locus (*hyp*) responsible for peptide auxotrophy in KS643. The location was corroborated by the relatively early entry of the hyp^+ marker in the interrupted matings with Hfr SA654 as well. This places the gene in the vicinity of the *rfb* and *galF* genes.

DISCUSSION

The *S. typhimurium* mutant KS643 described in this study has been shown to be unable to grow on simple minimal media. The results demonstrated that the required material for growth was a peptide and that the requirement was absolute. Studies on a variety of other microorganisms known to depend on the availability of peptides for growth (8, 19, 32, 34) led to two general conclusions: peptide-dependent growth in some amino acid auxotrophic organisms is due to the absence of the adequate transport system for the particular amino acid, and there is size restriction on peptide utilization above which peptides cannot enter the cell. The amino acid transport studies showed that all of the transport systems for amino acids were essentially intact in KS643, although activities varied somewhat. These results discard the rather weak possibility that the peptide requirement per se was simply due to an impaired transport system. Strain KS643 also would have had to harbor either a double mutation for amino acid auxotrophy and transport or a deletion through adjacent genes representing these functions, neither of which is particularly likely. Nutritional shiftdown does not cause immediate cessation of growth, but rather permits continuous cell divi-

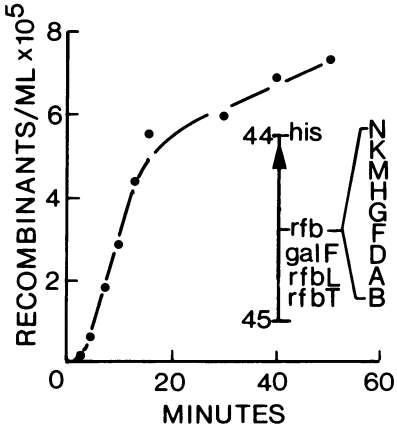

FIG. 5. *Localization of the hyp gene of S. typhimurium. The conjugation shown was of a cross between KS643 (F⁻) and SA535 (Hfr), and the point of entry of SA535 is shown in the inset with adjacent genes of interest.*

sion for three to four generations, with an apparent reduction in size. Multiple peptidase-deficient mutants of *S. typhimurium* which fail to carry out normal protein degradation show a prolonged lag phase after a nutritional shift-down, but eventually recover and grow on minimal medium (37).

The high hydrophobic nature of peptide P2 makes it unlikely that a peptide-mediated illicit transport of cations was correcting a cation transport problem, and treatment of the peptide with 0.2 M EDTA did not affect the activity. We have also observed that starvation for the peptide in various minimal media had no effect on the excretion of enterochelin, an iron-scavenging molecule, suggesting that iron transport was unaffected (unpublished data).

KS643 also showed a marked size preference for casein-derived peptides. Other peptides, such as trypsin inhibitor, bovine serum albumin, synthetic tripeptides, or acid-hydrolyzed commercial peptone, did not have any growth-promoting activity. We have also recently found that a combination of amino acids, as reflected by the composition of peptide P2, also will not stimulate growth.

The immunofluorescence studies on the interaction of the peptide with the bacterial membranes from SA33 and KS643 revealed a unique and interesting specificity; that is, the peptide was incorporated into or bound to the membrane only when grown in the presence of peptide and not by mere incubation of membrane particles with it. In other words, there appears to be a "growth specificity" which results from the interaction of peptides with the bacterial membrane. It is also possible that only right-side-out vesicles incorporate the peptide, but this has not been tested. These results do not indicate whether these relatively large peptides permeate both OM and IM. It is possible that immunologically reactive fragments are bound to the membranes. Alternatively, the whole peptides may possibly permeate the cell by partitioning in the lipid membrane.

The growth factor peptide has been partially characterized. It is known to be a neutral peptide of ca. 1,000 to 1,200 molecular weight, has a high hydrophobicity, and adsorbs to the membranes noncovalently. The absence of arginine or lysine residues in the peptide is perplexing since the commercial peptone from which it was derived was supposedly prepared by tryptic digestion. This should leave at least one basic residue per peptide, unless other methods were used and were not reported by the manufacturers. Alternatively, it may be that the active peptides are derived by tryptic action on the C-terminal ends of casein, releasing active C-terminal peptides. The data presented here do not necessarily demonstrate a causal relationship between the binding of the peptide to the membrane and the stimulation of growth. Since the peptide permeates the cell as well and supports growth, it may well be "correcting" an intracellular defect. However, our prejudice toward the correction of a membrane defect is based upon the known change in LPS structure to a new compositionally and immunologically distinct LPS still of the rough variety (Brãnes, Ph.D. thesis). One favored speculation is that either membrane fluidity or protein function is altered in association with the new LPS of KS643. There is ample evidence to support this supposition (3, 11, 23, 24, 31, 35), and the hypothesis has already been made that OM proteins undergo conformational changes or different packing arrangements or both depending upon the sugar composition of LPS core oligosaccharide in LPS mutants (6).

The only organism reported to date to require a specific peptide is the thermophilic acidophile *T. acidophilum* (31). There are remarkable similarities in the peptide requirement: *T. acidophilum* absolutely required a peptide component of yeast extract of molecular weight of about 1,000, other suitable peptides found in various peptones also sufficed, the growth response to this component was nearly identical to that demonstrated with KS643, and the activity of the peptide was also sensitive to N-terminal blocking. The main differences concern the composition and ion-binding properties. The amino acid composition of the *Thermoplasma* peptide was not particularly hydrophobic and had high amounts of basic and acidic residues. Its role in growth of *Thermoplasma* spp. was suggested to be due to its ion-scavenging properties, cell surface protection from high H^+ concentrations, ion transport capabilities, or possibly to supply essential amino acids in a form able to permeate the cells in its unusually acidic environment (31). Also, this ion-binding protein appeared to be associated with the *Thermoplasma* membrane (P. Smith, personal communication).

The unique pleiotropy of KS643 encompasses resistance to membrane antagonists, altered phage sensitivity, and disaccharide utilization, as well as peptide auxotrophy, although changes in some of these phenotypes have been noted with other OM mutants (2, 15, 26). Whether or not normal membrane function is restored by hydrophobic peptides still remains to be shown.

In this regard, there have been no discernible changes in the phospholipid, fatty acid, or protein composition, suggesting that the protein organization may be incorrect. Perhaps hydrophobic peptides correct this problem.

Although a detailed analysis of the genetic

defect in KS643 was not possible at this time, it is safe to say that the gene(s) coding for peptide auxotrophy resides near or in the *rfb* locus of the chromosome of *S. typhimurium*. Accurate analysis of this region was made difficult by the nature of the unusual phenotype of this strain which precluded following the usual type of genetic markers. The specific insertion sites of most Hfr's are not always known, and strain SA535 is only known to insert between the *his* operon and *metG*. This makes it possible that the mutation in KS643 may be associated with the *rfb* genes or perhaps with the *galF* gene(s). Recently, "partially rough" mutants of *Salmonella enteritidis* which were penicillin resistant and serum sensitive were mapped in the same region of the chromosome as KS643; however, this strain did not display the peptide auxotrophy described here (17).

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