JOURNAL OF BACTERIOLOGY, Oct. 1983, p. 308–315
0021-9193/83/100308-08$02.00/0

Vol. 156, No. 1

Intracellular Activation of Albomycin in *Escherichia coli* and *Salmonella typhimurium*

VOLKMAR BRAUN,* KLAUS GÜNTHNER, KLAUS HANTKE, AND LUITGARD ZIMMERMANN

Mikrobiologie II, Universität Tübingen, D-7400 Tübingen, West Germany

Received 28 April 1983/Accepted 18 July 1983

The antibiotic albomycin is actively taken up by *Escherichia coli* via the transport system for the structurally similar iron complex ferrichrome. Albomycin is cleaved, and the antibiotically active moiety is released into the cytoplasm, whereas the iron carrier moiety appears in the medium. Besides transport-negative mutants, additional albomycin-resistant mutants were isolated. The mutations were mapped outside the transport genes close to the *pyrD* gene at 21 min. The mutants were devoid of peptidase N activity. The molecular weight, sensitivity to inhibitors, and cytoplasmic location of the enzyme hydrolyzing albomycin in vitro corresponded to the known properties of peptidase N. The aminoacyl thioribosyl pyrimidine moiety of albomycin apparently has to be cleaved off the iron chelate transport vehicle to inhibit growth. Peptidase N is the major hydrolyzing enzyme. In *Salmonella typhimurium* peptidase N and peptidase A were equally active in hydrolyzing and activating albomycin.

The antibiotic albomycin is taken up by *Escherichia coli* (6–12, 15) and by *Salmonella typhimurium* (16) by the same transport system as ferrichrome. Mutations that lead to a transport-negative phenotype for *E. coli* were mapped in the *fhuA* (formerly *tonA*) gene and the closely linked *fhuB*, *fhuC*, (12) and *fhuD* (L. Fecker, unpublished data) genes. In addition, the functions specified by the *tonB* (7, 8) and *exbB* (9) genes are required for transport. There exists an additional class of albomycin-resistant mutants in *E. coli* which transport iron as ferrichrome complex with a normal rate. They could be impaired at the target site or in further processing of albomycin. Indeed, it was shown by differential radioactive labeling that albomycin is hydrolyzed by *E. coli*. Albomycin was either labeled chemically by replacing the *N*-acetyl groups by tritium-labeled acetyl groups or by growing the producing streptomyces strain in the presence of [^{35}S]sulfate (11). The ^{35}S-labeled portion and the iron remained in the cell, whereas the ^{3}H-labeled iron carrier moiety was excreted. It remained uncertain whether hydrolysis of albomycin is a necessary step for its antibiotic activity. We therefore studied additional albomycin-resistant, transport-active mutants to prove that albomycin is activated by sensitive cells. Some time ago we partially characterized such mutants. They showed in vitro an impaired hydrolysis of albomycin (K. Günthner, Diplomarbeit, Universität Tübingen, 1979; 10), and the mutations were all mapped close to the *pyrD* locus (L. Zimmermann, Diplomarbeit, Universität Tübingen, 1980). Because the first published chemical structure of albomycin was partially incorrect (26), it was difficult to imagine what kind of enzyme activity could hydrolyze it. Recently the structure was corrected (1), and it became immediately obvious that two peptide bonds could serve as potential cleavage sites (Fig. 1). Since the structural gene of peptidase N, *pepN*, maps close to *pyrD*, this enzyme was the most likely candidate. The major albomycin-hydrolyzing activity was eluted from a Bio-Gel P-100 column at a position corresponding to a molecular weight of 95,000, whereas aminopeptidase, probably identical to peptidase N, was supposed to consist of a single polypeptide chain with a molecular weight of 45,000 (14). These authors reevaluated their data and agree (Lazdunski, cited in reference 17) now with the molecular weight of 87,000 published recently (17). We show here that a certain class of albomycin-resistant mutants is devoid of peptidase N activity and that peptidase N-deficient mutants are conditionally albomycin resistant. We also demonstrate that in *S. typhimurium* peptidase N and peptidase A are equally active in converting albomycin to the antibiotically active form.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used were derivatives of *E. coli* K12 and of *S. typhimurium* LT2 and are listed in Table 1. Only typical representatives of the mutants isolated were included in the list.

Media and growth conditions. Cells were grown in

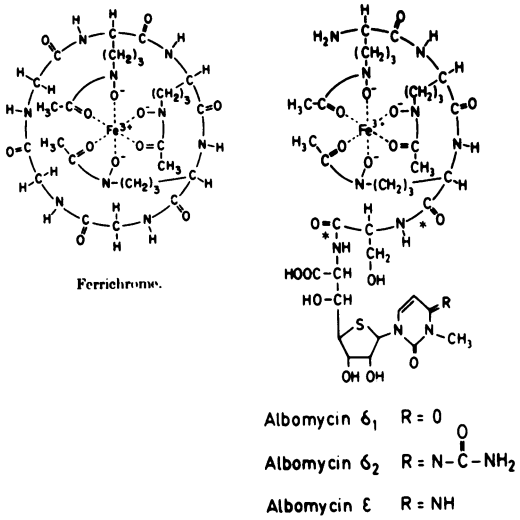

FIG. 1. Comparison of the structures of ferrichrome and albomycin (1). The asterisks indicate the suggested cleavage sites of the peptidases.

liquid culture or on agar plates containing either TY medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) or M9 salts (22) supplemented with 0.4% glucose, 1 mM sodium citrate, and 1 μg of thiamine per ml. If required the media contained (per ml) 20 μg of amino acids, except tryptophan, tyrosine, and phenylalanine (each 100 μg), and 40 μg of uracil, thymine, cytosine, 40 μM *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid. Iron-deficient plates contained 0.8% nutrient broth and 0.2 mM 2,2'-dipyridyl.

Genetic methods. For selection of albomycin-resistant mutants a culture of strain AB2847 in TY medium was mutagenized with phage Mu *cts* (19) and then grown at 30°C to the stationary phase. Cells (0.1 ml) were streaked on a TY plate onto which some drops of albomycin (0.1 mM) were placed. After incubation at 30°C resistant colonies were cross-streaked against albomycin.

Additional albomycin-resistant mutants of strain H455 were selected with the use of phage Mu *dl* (Ap *lac*) (3). After phage infection the cells were incubated for 3 h at 27°C in TY medium. Then albomycin (24 μg/ml) was added, and after 5 min cells were streaked on TY plates containing ampicillin (25 μg/ml) and incubated overnight at 27°C. Only those ampicillin- and albomycin-resistant mutants that were *lac*$^+$ on lactose MacConkey plates were studied further (19%). Spontaneous resistant mutants against albomycin were selected at 27°C on TY plates onto which a solution of 0.1 mM albomycin was dropped at one site.

Sensitivity of the albomycin-resistant derivatives was tested with phage T5 (*fhuA*), T1 (*fhuA tonB*), colicin M (*fhuA tonB exbB*), and colicin B (*tonB exbB*) (5). In addition, on iron-deficient plates transport-active derivatives showed a growth zone around filter paper disks impregnated with a solution of 1 mM ferrichrome (2).

Conjugation experiments and P1 transductions were performed by the method of Miller (22), and transductions with phage T4GT7 were performed as described previously (28). For each transduction the markers of 50 transductants were determined.

Biochemical methods. β-Galactosidase was determined as described by Miller (22). Identification of peptidase N-negative colonies followed the published procedure (19). However, 0.5 ml of ethanol was added to the solution of 2 mg of both L-alanyl-β-naphthylamide and Fast Garnet GBC (Sigma Chemical Co., St. Louis, Mo.) in 0.2 ml of dimethylformamide to obtain a homogeneous dye suspension upon the addition of 4.3 ml of 0.2 M Tris-hydrochloride (pH 7.5). Peptidase N activity in cell extracts was assayed by the method of McCaman and Villarejo (17).

Degradation of albomycin was determined with the supernatant fraction of a cell homogenate obtained with a French press (10,000 lb/in^2) and centrifuged for 1 h at 150,000 × *g*. We first used a 10 mM potassium phosphate buffer (pH 6.7), and we later used a 50 mM triethanolamine-hydrochloride buffer (pH 7.5). For the assays 10 mM NADH, 2 mM dipyridyl, and 10 mM $MgCl_2$ were added. Protein concentrations of 4.7 or 7.3 mg/ml were used. The plates contained 10 ml of 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.), M9 medium, 20 mg of 2,3,5-triphenyltetrazolium chloride per 100 ml, and 1.3 × 10^7 indicator cells. Wells (6-mm diameter) were filled with 20 μl of a solution of albomycin (0.7 μg/ml) or of the derived hydrolysis products. Triphenyltetrazolium chloride increased the accuracy of the determination of the diameter of the growth inhibition zone.

For the fractionation of the soluble cell extract, the supernatant fluid of 30 g of cells in 40 ml of 50 mM triethanolamine-hydrochloride buffer (pH 7.5)–10 mM $MgCl_2$ prepared as described above was used. Proteins were precipitated with 50% ammonium sulfate and redissolved in the above buffer. Nucleic acids were precipitated with protamine sulfate (0.08%) before 4 ml of the solution (20 mg of protein per ml) was applied to a Sephacryl S-200 superfine column K16/100, both from Pharmacia, Uppsala, Sweden. Proteins were eluted with the above buffer at a flow rate of 16 ml/h. Chromatography was monitored by determination of the UV absorbance and the conductivity. Samples of the fractions were tested for hydrolytic activity against deferrialbomycin. The four peaks that degraded albomycin were pooled separately. The same procedure was applied to separate proteins according to their size on a column filled with Bio-Gel P-100.

Chromatography of ^{35}S-labeled albomycin and its hydrolysis products on a column of Bio-Gel P-2 was done by A. Hartmann as described previously (11).

Chemicals. Albomycin was a gift of H. P. Fiedler, this institute. It consisted mainly of the δ2 compound. Bio-Gel P-100 and Bio-Gel P-2 were purchased from Bio-Rad, München, West Germany. Enolase (82,000), serum albumin (67,000), and catalase (240,000) (used as molecular weight standards) were obtained from Boehringer Mannheim.

All the other chemicals were of the highest purity available commercially.

RESULTS

Albomycin-resistant mutants. *E. coli* AB2847 mutagenized with Mu *cts* yielded among albomycin-resistant mutants 10% transport-active derivatives. The 10 derivatives, among which

TABLE 1. Strains used

Strain	Genotype and properties	Source or reference
E. coli K12		
AB2847	F⁻ *aroB thi tsx malT*	2
H1004	As AB2847, but *pepN*::Mu cts	This study
H1085	As AB2847, but *pepN*::Mu cts	This study
H455	As AB2847, but Δ(*pro-lac*)	6
ZI33	As H455, but *pepN*::Mu *d*1 (Ap *lac*)	This study
ZI90	As H455, but *pepN*::Mu *d*1 (Ap *lac*)	This study
ZI570	As ZI33, but pLM44	This study
ZI103	As H455, but spontaneous conditionally albomycin resistant	This study
W620	*pyrD trp his galK gltA6 rpsL*	U. Henning
ZI110	As W620, but *pepN*::Mu *d*1 (Ap *lac*)	This study
Hfr6	Hfr, *metB rel-1 mut-2 mtl-8*	B. Bachmann
Mal103	F⁻ Mu cts *d*1 (Ap *lac*) *araB*::Mu cts *araD139* Δ(*proAB-lacIPOZYA*) *rpsL*	3
MTM22	*pyrD zcb*::Tn*10 pepN308*	18
MTM110	*pepN pepA pepB pepQ pepD* Δ(*pro lac*)	M. McCaman
9200 and 9206	F⁻ *pepN arg trp his thr leu thi gal lac mal xyl mtl rpsL*	13
S. typhimurium		
TN87	*proB25 pepP1 pepQ1*	C. G. Miller
TN96	*proB25 pepP1*	C. G. Miller
TN98	*proB25 pepQ1*	C. G. Miller
TN852	*leuBCD485 pepN10 pepA1 pepB1 pepD3 zfg801*::Tn*10*	
TN858	*leuBCD485 pepN*⁺ *pepA1 pepB1 pepD3 zfg801*::Tn*10*	C. G. Miller
TN859	*leuBCD485 pepN10 pepA*⁺ *pepB1 pepD3 zfg801*::Tn*10*	C. G. Miller
TN860	*leuBCD485 pepN10 pepA1 pepB*⁺ *pepD3 zfg801*::Tn*10*	C. G. Miller
TN861	*leuBCD485 pepN10 pepA1 pepB1 pepD*⁺ *zfg801*::Tn*10*	C. G. Miller

H1004 and H1085 listed in Table 1 are typical representatives, exhibited the same unexpected resistance against high concentrations of albomycin (5 μM) on TY medium but partial sensitivity against low concentrations (0.1 μM) of albomycin on M9 medium. Logarithmically growing cells of the mutants in liquid culture continued growth for two generations in M9 medium after the administration of albomycin, whereas the fully sensitive parent strain immediately stopped growing at 0.01 μM albomycin. The addition of sugars, nucleosides, vitamins, or single amino acids to the M9 medium did not increase albomycin resistance. Only growth in the presence of 10% Casamino Acids rendered the mutants in M9 medium nearly as resistant as those in TY medium. This phenotype suggested a function involved in albomycin sensitivity that was suppressed in media with a high content of amino acids.

To study regulation, strain H455 *lac* (Table 1) was mutagenized with phage Mu *d*1 (Ap *lac*). When the phage is integrated into a gene in the orientation of its transcription, the lactose structural genes come under the control of that gene. Albomycin- and ampicillin-resistant mutants were obtained with a frequency of 5×10^{-7}. Of 200 mutants tested, 38 derivatives were Lac⁺. Of those, 24 were resistant to albomycin on TY medium but partially sensitive on M9 medium, and could grow on ferrichrome as the sole iron source (transport active). β-Galactosidase activity was the same (50 μmol of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per 10^9 cells per min at 28°C) in cells grown in TY medium or in TY medium rendered iron deficient by the addition of 200 μM dipyridyl. This showed that Mu was inserted in none of the known iron-regulated transport genes for albomycin (6).

Both Mu derivatives used as mutagens expressed a temperature-sensitive repressor that demanded growth of cells at temperatures below 31°C. We therefore tested whether the temperature was decisive in obtaining this new type of albomycin-resistant mutant. Of 50 spontaneous albomycin-resistant mutants of strain H455 selected at 37°C on TY plates, none showed the medium-dependent phenotype, whereas 7 conditional albomycin-resistant mutants were obtained at 27°C. When the latter were cross-streaked on M9 plates against a 0.4 μM albomycin solution, partial resistance was observed at 27°C, but sensitivity was observed at 37°C. The temperature dependence was also seen in TY liquid cultures with higher concentrations of albomycin (5 μM). At 27°C nearly identical growth curves were recorded with strain ZI101 in the presence and absence of albomycin, whereas at 37°C growth stopped after two generations with albomycin (data not shown). The same result was obtained with strain ZI570 Mu *d*1 (Ap *lac*), into which a

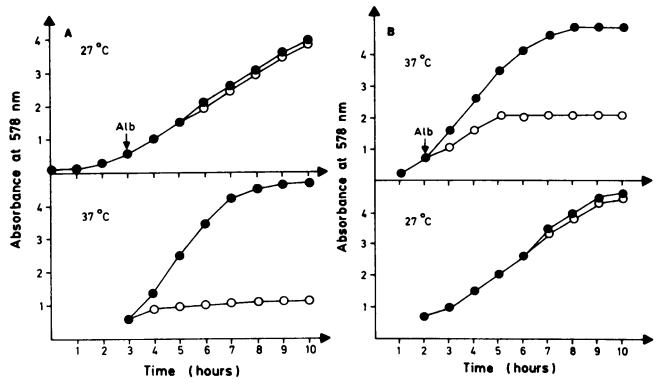

FIG. 2. Growth of *E. coli* ZI570 in TY medium in the presence (○) and absence (●) of albomycin. Albomycin (5 μM) was added after 3 h of growth, at which time the cultures were divided and cultivated further at 27 and 37°C, respectively. (A) Pregrowth at 27°C (upper panel) and shift to 37°C (lower panel); (B) pregrowth at 37°C and shift to 27°C.

temperature-resistant Mu repressor had been introduced by transformation with pLM44 (Fig. 2). Apparently the conditional albomycin resistance depends on the medium and the temperature. To see whether synthesis or the activity of a function conferring albomycin sensitivity was temperature dependent, cultures of strain ZI570 were shifted immediately after addition of albomycin from 27 to 37°C and vice versa. Cells became at once sensitive upon shift from 27 to 37°C (Fig. 2A) and resistant after the shift from 37 to 27°C (Fig. 2B).

Mapping of the conditional albomycin resistance marker. *E. coli* Hfr6, which transfers the chromosome clockwise starting at about 12 min, was conjugated with strain ZI110. Of 120 recombinants selected for *pryD*$^+$, 92 had become ampicillin and albomycin sensitive. Of 70 recombinants selected for *trp*$^+$, 32 were sensitive to the antibiotics, whereas all of the *his*$^+$ recombinants remained resistant. The result indicates a location of the albomycin resistance marker close to *pyrD*. Therefore, the cotransduction frequency between *pyrD* and the albomycin resistance marker was determined. Phage P1 and T4GT7 were grown on 10 of the Mu *d*1 (Ap *lac*) strains and on 7 of the spontaneous conditionally albomycin-resistant mutants. *E. coli* W620 *pyrD* served as the recipient strain. The cotransduction frequencies with Mu *d*1 (Ap *lac*) donor strains were between 12 and 56%; the average was 29%. Those of the spontaneous resistant donors were between 28 and 52%; the average was 40%. The transduction experiments supported the close linkage between *pyrD* and the albomycin resistance marker. The lower frequencies obtained with the Mu insertion mutants may arise from the extra 37 kilobases of DNA of the phage. Unexpectedly, we frequently observed a somewhat lower transduction frequency with T4GT7 than with P1, although the former phage can accommodate a larger piece of DNA (28).

Biochemical studies. We have previously shown that albomycin is cleaved intracellularly (11). However, the hydrolysis products were inactive against whole cells, probably because they could not enter the cells. We therefore assayed inactivation of albomycin in different cell fractions. The degradative activity of strain AB2847 was found in the soluble fraction; none was found in the membrane fraction. The supernatant fraction of strain H1085 grown on TY medium did not degrade albomycin. After growth on M9 medium there was some inactivation of albomycin. For example, after 2 h of incubation of 6 μg of albomycin per ml with a supernatant fraction (4.7 mg of protein per ml) of strain H1085, the zone of growth inhibition on plates (see above) was reproducibly reduced from 25 to 24 mm, whereas that obtained with the supernatant of AB2847 was reduced from 26 to 20 mm. Taking into consideration that the diameter of the inhibition zone depends on the logarithm of the albomycin concentration, the supernatant of AB2847 degraded 70% of albomycin, whereas that of H1085 destroyed at most 20%. Similar values were obtained with strain ZI33. Later we found out that hydrolysis of albomycin is much more effective when iron is removed by reduction with 10 mM NADH and incorporation into 2 mM dipyridyl. However, the relative values between the mutant and the parent and the medium dependence remained the same. Albomycin degradation thus corresponds qualitatively with albomycin sensitivity of cells.

To test whether there exists more than one albomycin-degrading activity, the soluble extract of cells grown in TY medium was chroma-

tographed on a column of Sephacryl S-200 superfine. Four activity peaks that degraded albomycin were eluted from the column. The elution positions corresponded to molecular weights in the range of 340,000, 95,000, 29,000, and 15,000 (data not shown). The major peak was that at 95,000.

Another gel permeation chromatography on Bio-Gel P-100 yielded two rather broad peaks; one was eluted with the void volume, and the other was eluted later. The first one probably contained the 340,000 and 95,000 fractions, and the second probably contained the 29,000 and 15,000 fractions. Both fractions were used to hydrolyze [^{35}S]albomycin. The chromatogram obtained with the first fraction (Fig. 3A) corresponded to the one obtained with [^{35}S]albomycin hydrolysis products released into the medium by sensitive cells (11). The rightmost peak corresponded in size to the ^{35}S-labeled hydrolysis product found in the cells (11). At longer incubation times this peak increased at the expense of albomycin and the middle peak (Fig. 3). Hydrolysis with the second fraction was less efficient (Fig. 3B) and yielded a mixture of compounds more heterogenous in size than did the fraction shown in Fig. 3A.

The albomycin-hydrolyzing activity of the major peak was inhibited by reagents that react with sulfhydryl groups such as 1 mM *p*-hydroxymercuribenzoate, 0.4 mM *N*-ethylmaleimide, *p*-chloromercuriphenylsulfonate, and $CuSO_4$. Activity could be restored by the addition of dithiothreitol; however, dithiothreitol also became inhibitory at concentrations above 3 mM. Typical inhibitors of serine proteases such as phenylmethylsulfonyl fluoride, *N*α-*p*-tosyl-L-lysine chloromethylketone, and L-1-tosylamide-2-phenylethyl chloromethylketone were at 0.2 mM concentrations without effect. The hydrolyzing activity for albomycin rapidly declined at pH values below 6.7.

Identification of peptidase N as major albomycin-cleaving enzyme in *E. coli*. When the correct structure of albomycin became known (1), it was immediately apparent that there were two potential peptide cleavage sites to release the sulfur-containing portion from the iron carrier moiety (Fig. 1). Inspection of the linkage map of *E. coli* revealed that *pepN*, the structural gene for peptidase N, maps close to *pyrD*, the locus we had identified for albomycin resistance.

The test for peptidase N activity for all of the conditional albomycin-resistant mutants was negative with colonies on plates; randomly selected mutants (ZI33, ZI90, ZI104, H1085, MTM22, MTM110, 9200, 9206) also showed no peptidase N activity in the cell extracts. Whereas the parent strain AB2847 yielded an absorbance of the released *p*-nitroanilide of 1.3 at 405 nm after 20 min, the absorbance obtained with the mutants was between 0.04 and 0.07.

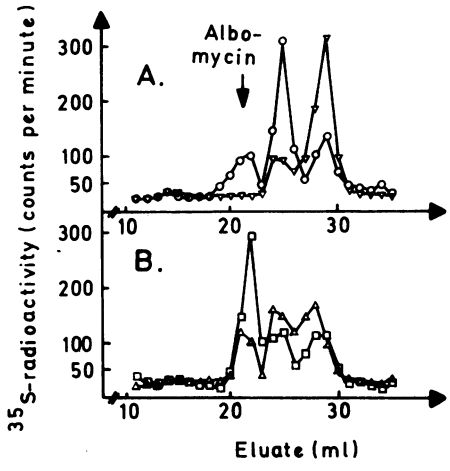

FIG. 3. Elution profile of cleavage products of 4 μM [δ_2-^{35}S]albomycin obtained after incubation for 2 h (○, □) and 5 h (▽, △) with (A) the high-molecular-weight fraction (peptidase N) and (B) the low-molecular-weight fraction of the gel filtration. The cleavage products were separated on a column of Bio-Gel P2 (60 by 0.9 cm) with a flow rate of 3 ml/h with water as the eluent. (Albomycin) marks the elution position of albomycin. The molecular weight standard trilysine was eluted at the position of the middle peak.

We tested the albomycin sensitivity of the *pepN* mutant MTM22 on TY and M9 plates and found a conditional resistance spectrum similar to that of our mutants. Mutant MTM22 was slightly more resistant on M9 medium than were our mutants. To test whether resistance changed with the genetic background of the strains, we transduced the tetracycline resistance marker of MTM22 *zcb*::Tn*10* into strain AB2847. Of 324 tetracycline-resistant colonies tested, 54% were *pepN* and conditionally albomycin resistant as the derivatives of AB2847 selected with albomycin. The functional peptidase N gene could be transduced back from an AB2847 *pepN*$^+$ *zcb*::Tn*10* derivative into strain H1004 *pepN* with a cotransduction frequency of 56% together with the Tn*10* marker. The *pepN*$^+$ derivatives were albomycin sensitive.

The multiple peptidase-negative mutant MTM110 *pepN pepA pepB pepQ pepD* was completely resistant to 5 μM albomycin on TY and M9 plates at 27°C and at 37°C. It became albomycin sensitive when *pepN*$^+$ was introduced in this strain together with *zcb*::Tn*10*. The cotransduction frequency was 49%.

The *pepN* strains 9200 and 9206 showed only slightly the medium and temperature dependence of albomycin resistance. They were largely resistant to albomycin also on M9 plates when

cross-streaked against a solution of 0.4 μM albomycin.

Activation of albomycin by *S. typhimurium*. We obtained a set of peptidase-negative mutants of *S. typhimurium* from C. G. Miller (Table 1). All of the mutants were albomycin sensitive, with the exception of the *pepN pepA* double mutants TN852, TN860, and TN861. Strains TN852, TN860, and TN861 exhibited no sensitivity in cross-streaks against 0.02 ml of a 1 μM albomycin solution on TY as well as on M9 plates. TN858 and TN859 were partially sensitive on TY plates and completely sensitive to the same extent (diameter of the clear zone of lysis) on M9 plates. To differentiate more between the *pepN*$^+$ *pepA* and the *pepN pepA*$^+$ mutants, growth of TN858 and of TN859 was measured in TY liquid medium containing 5 μM albomycin at 27°C and at 37°C and compared with the growth rates in the absence of albomycin. At 37°C strain TN859 did not grow in the culture containing albomycin. In the culture with TN858, the absorbance (turbidity) increased in 5 h from 0.1 to 0.5; during this time the albomycin-free cultures of both strains reached an absorbance of 4.3. At 27°C for both strains parallel growth curves were obtained that reached an absorbance of 1.5 with albomycin and of 2.6 without albomycin after 5 h of cultivation. Strain TN859 was again slightly more sensitive to albomycin than was TN858. These data demonstrate that in *S. typhimurium* peptidase A is as active as peptidase N in rendering cells sensitive to albomycin. The *S. typhimurium* strain also shows a temperature dependence similar to that observed in *E. coli* (Fig. 2).

DISCUSSION

The parent strain *E. coli* AB2847, from which the albomycin-resistant mutants were isolated, could not synthesize its own iron chelator enterochelin because of the mutation in the *aroB* gene. Under iron-limiting conditions in the presence of 0.2 mM dipyridyl, growth was dependent on the added siderophore ferrichrome. Thus transport-deficient, albomycin-resistant mutants could clearly be differentiated from transport-independent, albomycin-resistant mutants. The puzzling phenotype of the mutants, resistance on rich TY medium, partial resistance at 27°C, and sensitivity at 37°C on minimal medium, could be solved when peptidase N was identified as the major albomycin-activating enzyme. The data can now be reconciled with the following concept. Albomycin has to be cleaved into the iron carrier moiety and the aminoacylthioribosyl pyrimidine part. As shown previously (11) the iron stays in the cells, the carrier is excreted like deferri-ferrichrome (10), and the aminoacylthioribosyl pyrimidine moiety remains inside and is antibiotically active. Mutants devoid of peptidase N are albomycin resistant on rich medium. Albomycin is taken up because it promotes growth on iron-deficient nutrient broth plates containing dipyridyl (data not shown). On rich medium peptidase N is active. It is not regulated by iron and is synthesized constitutively (18). However, there are conflicting results in the literature about the regulation of *pepN* by phosphate (18, 23). A second, lower activity for albomycin hydrolysis is seemingly suppressed on rich media. It is expressed on minimal media. At 27°C its activity is so low that at moderate concentrations of albomycin, which are still more than 100 times above the minimal inhibitory concentrations (0.01 μM) of the parent strain, *pepN* mutants are resistant. At 37°C *pepN* mutants become sensitive. This phenotype may reflect the usual temperature dependence of the turnover rate of substrates determined by the enzyme activity and substrate concentration. Since two enzymes, peptidase N and peptidase A, were equally effective in conferring albomycin sensitivity to *S. typhimurium*, the temperature dependence of albomycin activity revealed by the growth inhibition studies in liquid culture probably reflected mostly the albomycin concentration in the cells.

It seems that the lower transport rate at 27°C determined the conditional albomycin sensitivity of the *E. coli pepN* mutants and of the *S. typhimurium pepN pepA* mutants. This conclusion is supported by the finding that albomycin-resistant *E. coli pepN* mutants became albomycin sensitive when they were transformed with multicopy plasmids carrying the entire region of the *fhu* transport genes (L. Fecker and V. Braun, unpublished data). The increase in transport capacity apparently enhanced albomycin concentration in the cell to a level that the less efficient peptidases provided sufficient amounts of antibiotically active albomycin hydrolysis product(s) to inhibit cell growth.

Evidence for additional peptidases in *E. coli* that can hydrolyze and thus activate albomycin came from the four peaks that were obtained when the soluble fraction of cells was separated by gel permeation chromatography on Sephacryl S-200 superfine. The size of the major component was determined to be 95,000, which corresponds to the molecular weight of peptidase N of 87,000 (17). Sensitivity of the 95,000 protein fraction to various chemicals also corresponded to that of peptidase N (17). The second enzyme important in vivo for albomycin sensitivity was found to be peptidase A in *S. typhimurium*. The corresponding *E. coli* enzyme was formerly suggested to be identical with aminopeptidase I (20), characterized by Vogt (27). Its molecular

weight of 320,000 is, within the accuracy of the determination we employed, similar to the size of 340,000 of the enzyme(s) in the first elution peak from the Sephacryl column. This enzyme activity also eluted from a DEAE-Sepharose column at an ionic strength of 0.04 M KCl (data not shown), as does aminopeptidase I (27). Furthermore, peptidase A exhibits a broad substrate specificity similar to that of peptidase N (17, 19–21, 27), so that both enzymes are the most likely candidates of the peptidases characterized biochemically to be able to cleave albomycin. The activity of peptidase A in *E. coli* K12 seems to be low compared to the activity in *S. typhimurium*. However, these activity differences could also reflect different albomycin concentrations in the two cell types. The additional activities found in the in vitro assay may be too low to be relevant for cell inhibition at the rather small albomycin concentrations used to avoid side effects. Peptidase N activity can be determined in whole cells and cell extracts by assays that are specific for peptidase N (13, 14, 17–21, 24). The enzyme is easily accessible for its substrate from outside in whole cells; this had been one of the arguments for a cytoplasmic membrane association of the peptidase N (24), which otherwise behaves like a soluble cytoplasmic protein (17). This localization would support the idea that the cleavage of albomycin should take place near or in the cytoplasmic membrane to allow excretion of the iron carrier moiety and accumulation of the antibiotically active compound in the cytoplasm.

Albomycin contains four peptide bonds that could be potential cleavage sites for peptidase (Fig. 1). The finding that the iron-free form is cleaved much more rapidly in vitro than the iron-containing molecule suggests that in cells iron is released before activation occurs. It is possible that the iron-free molecule constitutes a linear peptide that is cleaved successively starting from the amino-terminal end. However, we have isolated from the culture medium of albomycin-sensitive *E. coli* cells radioactive hydrolysis products of distinct size (11). Albomycin was either labeled chemically by replacing the *N*-acetyl groups by tritium-labeled acetyl groups or by growing the albomycin-producing strain *Streptomyces griseus* Tü6 in the presence of [^{35}S]sulfate (11). A single tritium-labeled compound was released from the cells which contained the three *N*-hydroxy-*N*-acetyl ornithine residues forming the ligand around the iron (Fig. 1). Since the peptide bonds between the ornithine derivatives are not hydrolyzed, preferential cleavage sites must occur in the region joining the iron ligand carrier to the aminoacylthioribosyl pyrimidine derivative. If the serine residue joining the aminoacylthioribosyl pyrimidine residue to the iron ligand is cut out, the remaining pyrimidine derivative still has a molecular weight of 388. The reason for its separation from the iron ligand may be sought in the pyrimidine moiety because such compounds have a tendency to adsorb to the gel matrix, resulting in a delayed elution. The intermediary product of the ^{35}S-labeled moiety released from sensitive cells emerges at the trilysine position but is converted to the smaller final product, which is the only one retained by the cells (11). The same elution profile was obtained from the in vitro hydrolysis of [^{35}S]albomycin by a cell fraction containing peptidases A and N (Fig. 3A).

Separation according to differences in size is certainly not adequate to determine the cleavage sites of albomycin, but the identity in the elution profiles and the conversion of albomycin through an intermediate to the final product obtained in vivo and in vitro suggest that the two enzymes act as major activators of albomycin. The genetic evidence proves that one of them has to be active to render cells sensitive to albomycin. However, the data do not exclude the possibility that additonal functions are involved in converting albomycin to an antibiotic. Studies are underway to hydrolyze albomycin with purified peptidase N to identify the target and to unravel its mode of action with the antibiotically active moiety.

There are other antibiotics containing carriers that have to be cleaved to activate the antibiotic (4, 25, 29). In most of them the antibiotically active moieties are linked to peptides and are actively taken up via the peptide transport systems. The inhibitor may also be an amino acid analog or may be as distinct from the carrier as that of albomycin. Ferrimycin A1, which contains the iron carrier ferrioxamine B, is analogous to ferrichrome and albomycin. The antagonism between these compounds rests on competition for the same transport system. Since this siderophore-sideromycin pair also contains an amide bond that links the iron carrier to the inhibitor, it is likely that a peptidase activates intracellularly ferrimycin A1.

ACKNOWLEDGMENTS

We thank M. T. McCaman, C. G. Miller, and C. Lazdunski for providing us with strains and H.-P. Fiedler for the gift of albomycin. The excellent technical contributions of Helga Wolff are gratefully appreciated.

This research was supported by the Deutsche Forschungs gemeinschaft (grant SFB 76).

LITERATURE CITED

1. **Benz, G., T. Schröder, W. Karl, T. Kurz, J. Pfitzner, D. Schmidt, G. Steffens, and C. Wünsche.** 1982. Konstitution der Desferriform der Albomycine. Angew. Chem. **94**:552–553.

2. **Braun, V., and R. Burkhardt.** 1982. Regulation of the ColV plasmid-determined iron (III)-aerobactin transport in *Escherichia coli*. J. Bacteriol. **152**:223–231.
3. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using Mu-*lac* bacteriophage: in vivo probe for transcriptional control sequences. Proc. Natl. Acad. Sci. U.S.A. **76**:4530–4533,
4. **Chopra, I., and P. Ball.** 1982. Transport of antibiotics into bacteria. Adv. Microbiol. Physiol. **23**:184–240.
5. **Davies, J. K., and P. Reeves.** 1975. Genetics of resistance to colicins in *Escherichia coli* K-12: cross-resistance among colicins of group B. J. Bacteriol. **123**:96–101.
6. **Hantke, K.** 1981. Regulation of ferric iron transport in *Escherichia coli* K-12: isolation of a constitutive mutant. Mol. Gen. Genet. **182**:288–292.
7. **Hantke, K., and V. Braun.** 1975. Membrane receptor dependent iron transport in *Escherichia coli*. FEBS Lett. **49**:301–305.
8. **Hantke, K., and V. Braun.** 1978. Functional interaction of the *tonA/tonB* receptor system in *Escherichia coli*. J. Bacteriol. **135**:190–197.
9. **Hantke, K., and L. Zimmermann.** 1981. The importance of the *exbB* gene for vitamin B12 and ferric iron transport. FEMS Microbiol. Lett. **12**:31–35.
10. **Hartmann, A., and V. Braun.** 1980. Iron transport in *Escherichia coli*: uptake and modification of ferrichrome. J. Bacteriol. **143**:246–255.
11. **Hartmann, A., H. P. Fiedler, and V. Braun.** 1979. Uptake and conversion of the antibiotic albomycin by *Escherichia coli* K-12. Eur. J. Biochem. **99**:517–524.
12. **Kadner, R. J., K. Heller, J. W. Coulton, and V. Braun.** 1980. Genetic control of hydroxamate-mediated iron uptake in *Escherichia coli*. J. Bacteriol. **143**:256–264.
13. **Latil, M., M. Murgier, A. Lazdunski, and C. Lazdunski.** 1976. Isolation and genetic mapping of *Escherichia coli* aminopeptidase mutants. Mol. Gen. Genet. **148**:43–47.
14. **Lazdunski, C., J. Busutill, and A. Lazdunski.** 1975. Purification and properties of a periplasmic aminopeptidase from *Escherichia coli*. Eur. J. Biochem. **60**:363–369.
15. **Leong, J., and J. Neilands.** 1976. Mechanisms of siderophore iron transport in enteric bacteria. J. Bacteriol. **126**:823–830.
16. **Luckey, M., J. R. Pollack, R. Wayne, B. N. Ames, and J. B. Neilands.** 1972. Iron uptake in *Salmonella typhimurium*: utilization of exogenous siderophores as iron carriers. J. Bacteriol. **111**:731–738.
17. **McCaman, M. T., and M. R. Villarejo.** 1982. Structural and catalytic properties of peptidase N from *Escherichia coli* K-12. Arch. Biochem. Biophys. **213**:384–394.
18. **McCaman, M. T., A. McPortland, and M. R. Villarejo.** 1982. Genetics and regulation of peptidase N in *Escherichia coli* K-12. J. Bacteriol. **152**:848–854.
19. **Miller, C. G.** 1975. Genetic mapping of *Salmonella typhimurium* peptidase mutations. J. Bacteriol. **122**:171–176.
20. **Miller, C. G., and K. Mackinnon.** 1974. Peptidase mutants of *Salmonella typhimurium*. J. Bacteriol. **120**:355–363.
21. **Miller, C. G., and G. Schwartz.** 1978. Peptidase-deficient mutants of *Escherichia coli*. J. Bacteriol. **135**:603–611.
22. **Miller, J. H.** 1982. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Murgier, M., and S. Gharbi.** 1982. Fusion of the *lac* genes to the promoter for the aminopeptidase N gene of *Escherichia coli*. Mol. Gen. Genet. **187**:316–319.
24. **Murgier, M., C. Pelissier, A. Lazdunski, A. Bernadac, and C. Lazdunski.** 1977. Aminopeptidase N from *Escherichia coli*: unusual interactions with the cell surface. Eur. J. Biochem. **74**:425–433.
25. **Ringrose, D. S.** 1980. Peptides as antimicrobial agents, p. 641–692. *In* J. W. Payne (ed.), Microorganisms and nitrogen sources. Transport and utilization of amino acids, peptides, proteins and related substrates. John Wiley & Sons, Inc., New York.
26. **Turkova, J., O. Mikes, and F. Sorm.** 1966. Similarities in the composition of antibiotics albomycin and grisein. Collect. Czech. Chem. Commun. **31**:2444–2453.
27. **Vogt, V. B.** 1970. Purification and properties of an aminopeptidase from *Escherichia coli*. J. Biol. Chem. **245**:4760–4769.
28. **Wilson, G., K. K. Y. Young, and G. J. Edlin.** 1979. High frequency generalised transduction by bacteriophage T4. Nature (London) **280**:80–82.
29. **Zähner, H.** 1977. Some aspects of antibiotic research. Angew. Chem. Int. Ed. Engl. **16**:687–694.