Relationship Between Permeability, Cell Division, and Murein Metabolism in a Mutant of *Escherichia coli*

CLAUDE LAZDUNSKI AND BENNETT M. SHAPIRO

Department of Biochemistry, University of Washington, Seattle, Washington 98195

A mutant of *Escherichia coli* has been found to have an increased sensitivity to actinomycin D and to sodium deoxycholate and an unusual morphology which accompanies an abnormality in cellular division. All of these characteristics are suppressed when the strain is grown in the presence of D-alanine. This strain, called MAD-1, for murein altered division mutant, exhibits its pleiotropic phenotype only when certain carbon compounds are used as energy sources in minimal medium. Nonpermissive carbon sources, which elicit the disturbed phenotype, include glucose, mannitol, fructose, maltose, and lactose; permissive carbon sources include galactose, glycerol, lactate, and succinate. The mutant is able to transport nonpermissive carbon compounds; 3 mM 3',5'-cyclic adenosine monophosphate included in the medium does not alter the phenotype seen with growth on glucose. Deoxyribonucleic acid and protein synthesis are normal with respect to cellular mass increase. D-Alanine specifically suppresses the pleiotropic phenotype at a concentration six times lower than L-alanine, the only other compound found to be effective. There is no abnormality in the K_m or V_{max} of L-alanine racemase or D-alanine-D-alanine synthetase of MAD-1 compared to its parent, CR34. MAD-1 is more susceptible to growth inhibition by penicillin or cycloserine than its parent, and is exquisitely sensitive to lysis in the presence of sodium deoxycholate or lysozyme. When cell wall biosynthesis is inhibited, MAD-1 lyses much more rapidly than CR34, even after it has been phenotypically suppressed by growth on D-alanine. The incorporation of L-alanine and diaminopimelic acid into the peptidoglycan of the mutant and wild type is identical; D-alanine is incorporated 1.5 times more rapidly into MAD-1 cells grown under nonpermissive conditions. The peptidoglycan fragments seen after digestion with lysozyme were similar for MAD-1 and the wild type. The results are interpreted as being compatible with an increased autolytic rate in MAD-1, caused either by an increase in the quantity or activity of an autolysin, or by an abnormal cell wall which is especially susceptible to autolysis, but which was not detected by analysis of peptidoglycan fragments.

Because of increasing lines of evidence which suggest that the bacterial surface is involved in the cell division process (reviewed in 4, 5), we have selected for surface-altered bacterial mutants and investigated whether or not they are affected in cell division. In the accompanying paper we describe one such selection technique (7), that of looking for mutants of *Escherichia coli* K-12 with increased permeability. One of the mutants thus found (strain 2404) has been shown to have an abnormal morphology when grown on certain carbon sources. This strain is called MAD-1, as an acronym for murein altered division mutant. MAD-1 is phenotypically wild type when growing on nutrient medium, but when growing on minimal medium with glucose or certain other carbon sources, it exhibits an increased cellular mass and a bizarre morphology. This paper describes some physiologic properties of this mutant and suggests that the defect in this strain is an increase in autolysis which leads to both its increased permeability and its cell division abnormality.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strain 2404 was isolated as previously described (7) from CR34, which is $F^- leu^- thr^- thi^- lac Y^- Sm^s$. This mutant is also called MAD-1 in this investigation.

Media and growth of bacteria. The media are the same as in the accompanying paper (7).

Macromolecular biosynthesis. L-Leucine-*U*-^{14}C (260 mCi/mmole) and thymine-2-^{14}C (30 mCi/mmole) were purchased from New England Nuclear Corp. Incorporation of ^{14}C-leucine and ^{14}C-thymine into trichloroacetic acid-insoluble material was used as an index of protein and deoxyribonucleic acid (DNA) synthesis, respectively. The radioactive precursors were added to exponentially growing cultures of mutant or wild-type bacteria; at intervals after the addition of the radioactive precursors, 0.6-ml samples of culture were removed to 1 ml of ice-cold 10% trichloroacetic acid containing 300 μg of L-leucine or thymine per ml to stop incorporation. After 30 min at 0 C the acid-insoluble precipitates were collected on membrane filters (0.45 μm; Millipore Corp.) and washed three times with 3 ml of ice-cold trichloroacetic acid (5%) containing 100 μg of leucine or thymine per ml, respectively. Then, they were washed with 3 ml of 0.01 N HCl. After drying, the filters were placed in 10 ml of Liquifluor (New England Nuclear Corp.) scintillation mixture for counting in a Beckman liquid scintillation counter.

Cell number. The cell number was determined by direct counting with the Hauser-Petroff bacterial counter. The number of colony-forming units was measured by serial dilution of the culture and plating on nutrient agar. To compare the number of bacteria per unit mass under different growth conditions, we have defined a quantity, V, which is the number of cells per milliliter per unit of optical density at 600 nm. This number V is inversely proportional to the average size of the bacterial cells in the population, for the optical density is proportional to the mass of the bacterial culture. Thus, a small value of V is indicative of relatively larger cells in the bacterial population.

Uptake experiments. The kinetics of uptake of several carbon sources used for growth were measured by using ^{14}C-labeled sugars. The cells were pregrown on the carbon source to be tested. After washing exponentially growing cells with 63B₁ medium, they were rapidly resuspended in 2.5 ml of 63B₁ containing chloramphenicol (60 μg/ml) and the ^{14}C-labeled carbon source (0.11 mM). At intervals, 0.5-ml samples were taken, rapidly filtered onto Whatman GF/C discs (2.5 cm), and washed twice with 3 ml of ice-cold 63B₁ medium. The filtration and washing took less than 10 sec. The presence or absence of 10 mM sodium azide in the reaction mixture had no effect on the kinetics. All of these uptake experiments were at 30 C.

Enzyme assays. For all enzyme assays, cell extracts were prepared by sonic disruption at 0 C using the micro tip of a Biosonic III sonic oscillator, with exposure to 30 w/cm² for 2 min. Cell debris was removed by centrifugation at 30,000 × *g* for 20 min.

Hexokinase was assayed by following the formation of reduced nicotinamide adenine dinucleotide phosphate (NADH), in the procedure of DiPietro and Weinhouse (2), at 30 and 40 C. L-Alanine racemase was assayed in a coupled assay employing D-amino acid oxidase as described by Julius et al. (6). D-Alanine-D-alanine synthetase was assayed by following the conversion of isotopically labeled ^{14}C-D-alanine into the dipeptide D-alanine-D-alanine by chromatography, as described by Neuhaus (9).

Other procedures. The morphology of the cells was determined by phase-contrast microscopy using a Zeiss microscope and oil immersion objectives. Cell walls were prepared for examination by the procedure of Schwarz et al. (10), and mucopeptides were prepared as described by Leutgeb and Schwarz (8), by digestion of cell wall with lysozyme and subsequent chromatographic separation of the different peptidoglycan fragments.

RESULTS

Growth properties. The mutant MAD-1 grows similarly to wild type on nutrient medium; that is, there is only a 20% increase in the generation time for the mutant, and the mutant and the wild type have the same rod morphology. Depending on the carbon compound used in minimal growth medium, there is an abnormal morphology and slower generation time for the mutant strain. A typical carbon source which evokes the morphologic alteration is glucose, and Fig. 1 illustrates the morphology of MAD-1 growing on glucose at 40 C. At 30 C, MAD-1 appears coccoid; whereas, at 40 C, MAD-1 is heterogeneous, with forms ranging from coccal to large heteromorphic masses several times the size of a normal *E. coli* (Fig. 1A, B). Each of these forms can give rise to a colony if plated on nutrient agar, for the number of visible bacteria (when all particles are counted in one of these heteromorphic preparations) is the same as the number of viable bacteria, as determined by colony formation upon subsequent dilution and plating on nutrient agar. However, there are fewer colonies (or cells) per unit of optical density under these growth conditions than when MAD-1 is growing on nutrient agar, or when the wild type is growing on nutrient agar or on glucose-63B₁ medium. We have used V, the ratio of the number of colony-forming bacteria divided by the optical density, to illustrate the extent of the abnormality in semiquantitative terms. A large value of V indicates a larger number of colony-forming units per unit mass.

Table 1 illustrates the response of MAD-1 to a number of different carbon compounds used as energy sources in minimal medium. It is
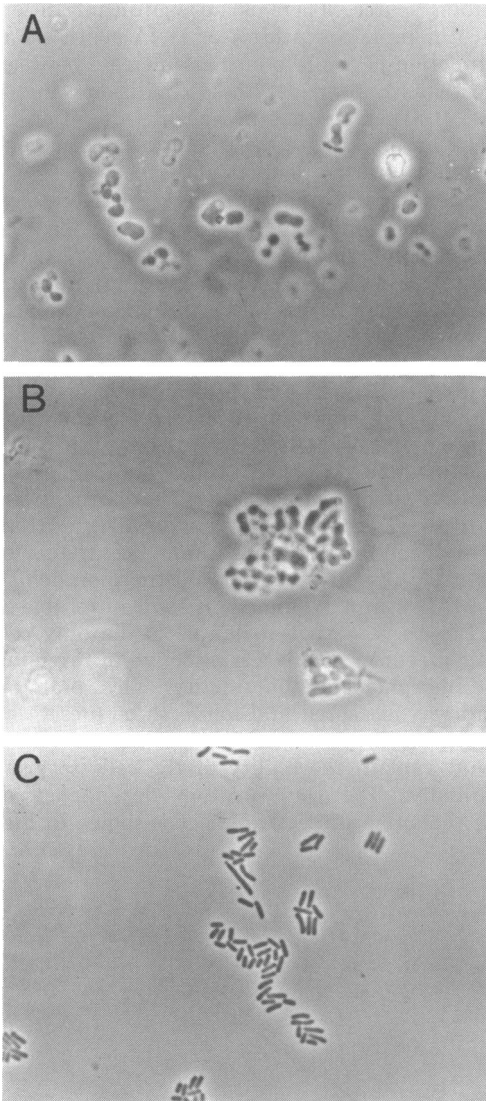
FIG. 1. *Effect of the composition of the growth medium upon the morphology of MAD-1. Phase-contrast microscopy was used to examine MAD-1 growing on agar-coated slides at 40 C. The media were glucose-minimal medium (A and B) and nutrient agar (C). In B there is a large microcolony which illustrates the pleiomorphic cells arising from a single cell. All bacteria were growing exponentially on the same liquid medium as the agar slides before plating on agar; the morphology is the same in liquid as in solid media. Magnification is ×2,000.*

TABLE 1. *Growth properties of MAD-1 and CR34 (wild type) on different carbon compounds at 40 C*

Carbon compound	Generation time[a] (min)		V^b (cells) (MAD-1)	Morphology[b] (MAD-1)
	CR34	MAD-1		
Glucose	66	110	52×10^7	Abnormal
Galactose	120	130	7×10^8	Normal
Glycerol	110	140	7×10^8	Normal
Lactate	110	120	5×10^8	Normal
Succinate	125	150	8×10^8	Normal
Mannitol	100	100	4.5×10^8	Abnormal
Fructose (30 C)	110	125	8×10^7	Abnormal
Maltose	85	104	1×10^7	Abnormal
Lactose	—	260	3.5×10^8	Abnormal

[a] The generation time for CR34 on nutrient medium was 42 min and for MAD-1 was 50 min. Under these conditions, both had a value of 7×10^8, and both had normal morphology.

[b] In all cases, the V for CR34 was 7×10^8 to 8×10^8 cells per optical density unit at 600 nm, and the morphology was that of a homogeneous rod population.

strain (CR34) grows on glucose with a generation time of 66 min, whereas MAD-1 has a generation time almost double that (110 min). CR34 produces 7×10^8 colony-forming units per unit of optical density, whereas MAD-1 has about 8% of the number of cells or colony-forming units per unit mass. The abnormal morphology of MAD-1 on glucose and the other nonpermissive carbon sources is that illustrated by Fig. 1B. Permissive carbon compounds for growth in minimal medium include galactose, glycerol, lactate, and succinate, on all of which the generation time of MAD-1 is only slightly longer than that of the wild type and the number of colony-forming units per unit of optical density is approximately the same as that of the wild type. On fructose, maltose, and mannitol, the value of V is decreased and the morphology is abnormal. It is interesting that, in mannitol, the value of V is not markedly decreased, but there is an abnormal morphology. The morphology on mannitol is not quite as bizarre as that on glucose. CR34 does not grow on lactose with any appreciable generation time, since it is a permease-less mutant, but MAD-1 does grow, although slowly, with an abnormal morphology and with a V value of half of what it is on permissive carbon sources. We have been unable to reverse the effects of growth on $63B_1$-glucose by the addition of 3',5'-cyclic adenosine monophosphate (AMP) at a concentration of 3 mM.

Sugar uptake. To investigate further the

clear from this table that one can identify two classes of carbon compound: those which are permissive and those which are nonpermissive for normal morphology and normal cell division in MAD-1. For example, the wild-type

relationship between the carbon source used for growth and the cell division and morphologic abnormality, we measured the uptake for several different permissive and nonpermissive carbon sources. The kinetics of uptake by the mutant strain for glucose, galactose, succinate, and fructose are similar to that of parental strain, CR34 (Fig. 2). In fact, after growth at 40 C, the mutant seems to transport glucose and galactose even better than the wild type. Thus, the morphologic abnormality does not seem to be related to the ability of the cells to take up the nonpermissive carbon source. In addition, hexokinase was assayed in the mutant and wild-type strains grown at 30 or 40 C, and there was no difference in the activity of the enzyme. We still do not understand the reason for the carbon source dependence of the morphologic abnormality, but some possible interpretations are presented in the discussion section.

Macromolecular biosynthesis. To see whether macromolecular biosynthesis was specifically affected in the mutant growing on nonpermissive carbon sources, we investigated the incorporation of leucine into protein and thymine into DNA. Mutant and wild-type strains were grown at 40 C on $63B_1$, supplemented with lactate, a permissive carbon source for MAD-1, or glucose, a nonpermissive

carbon source. There is no significant difference in protein synthesis or DNA synthesis as a function of the rate of mass increase between the two strains under these conditions (Fig. 3 and 4). There is thus no specific alteration in DNA or protein synthesis in MAD-1 on nonpermissive carbon sources.

Phenotypic correction. Since MAD-1 grows normally on nutrient medium but not on glucose-minimal medium, the possibility existed that there was some constituent of the nutrient medium which was able to phenotypically suppress the effects of the mutation. Initially, we tried supplementation with Casamino Acids or purines and pyrimidines, or both, without any effect on reversing the abnormal morphology of MAD-1 growing on $63B_1$-glucose medium. We then tried a number of cell wall precursors, with the idea that perhaps one of these was a limiting factor during growth of MAD-1 on minimal medium. The effects of several cell wall precursors on the morphology of MAD-1 in $63B_1$-glucose is shown in Table 2. It can be seen that D-alanine is the only cell wall precursor which was effective in reversing the morphologic abnormality. This D-amino acid was measured and found to be present at 70 μg/ml in our nutrient medium and thus could explain the ability of the cells to grow normally. The concentration dependence of the D-alanine protective effect is shown in Fig.
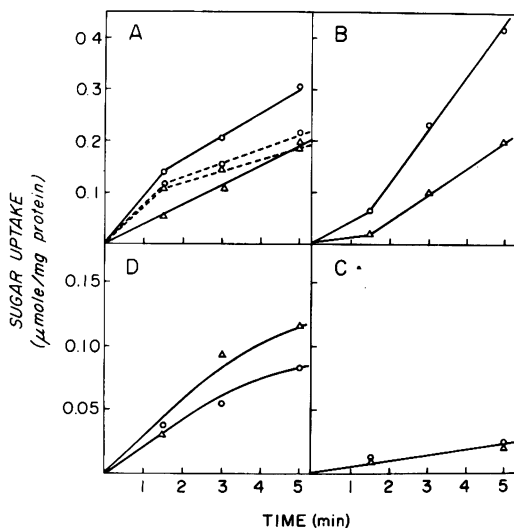

FIG. 2. *Kinetics of uptake of permissive and nonpermissive carbon sources in MAD-1 and its parent. The uptake of glucose (A), galactose (B), succinate (C), and fructose (D) were measured for CR34 (△) or MAD-1 (○) grown on minimal medium containing the same carbon compound at 30 C (----) or at 40 C (——). All incorporation experiments were at 30 C.*
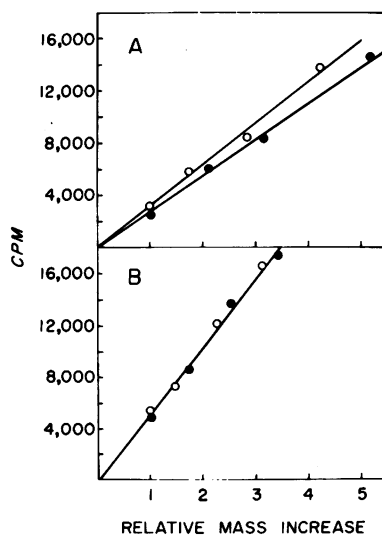

FIG. 3. *Protein synthesis in MAD-1 and its parent. CR34 (●) and MAD-1 (○) were grown in $63B_1$ medium supplemented with lactate (A) or glucose (B) at 40 C, and the incorporation of ^{14}C-leucine as a function of mass increase was measured as described by Friesen (3).*
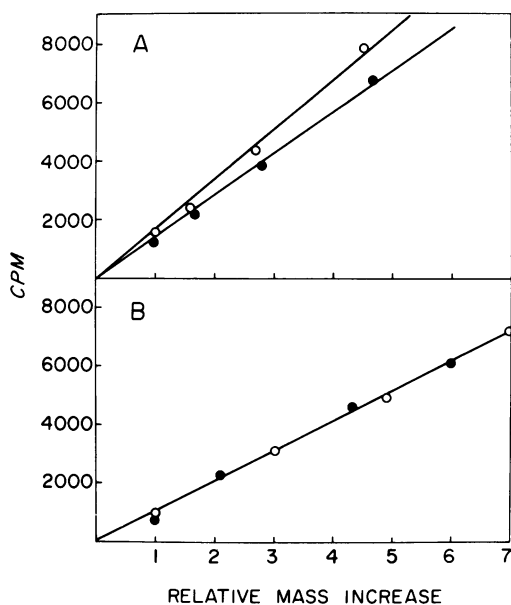
FIG. 4. DNA synthesis in MAD-1 and its parent. CR34 (●) and MAD-1 (○) were grown on 63B₁ medium supplemented with lactate (A) or glucose (B) at 40 C, and the uptake of ¹⁴C-thymine into DNA was measured as described by Friesen (3).

TABLE 2. Effect of cell wall precursors on the morphology of MAD-1 growing on 63B₁-glucose (0.4%) at 40 Cᵃ

Compound	Morphology
m-Diaminopimelate	Abnormal
D-Glutamate	Abnormal
D-Alanine	Normal
Glucosamine	Abnormal
Muramic acid	Abnormal

ᵃ All precursors were present in the medium at 150 μg/ml. Abnormal morphology is like that of Fig. 1A or B; normal morphology is like that in Fig. 1C, which is essentially that of the parent.

5. In this figure, the value V is plotted as a function of D-alanine or L-alanine concentration, thus permitting quantitation of the effect of the phenotypic suppressors. D-Alanine half-maximally suppresses the MAD-1 division abnormality at concentration of 75 μg/ml, whereas L-alanine is effective only at a concentration some six times higher. The ability of both L- and D-alanine to reverse the cell division abnormality thus led us to suspect that there might be a defect in the L-alanine racemase, which would result in a decreased level of D-alanine under certain growth conditions. We very carefully examined both the K_m and
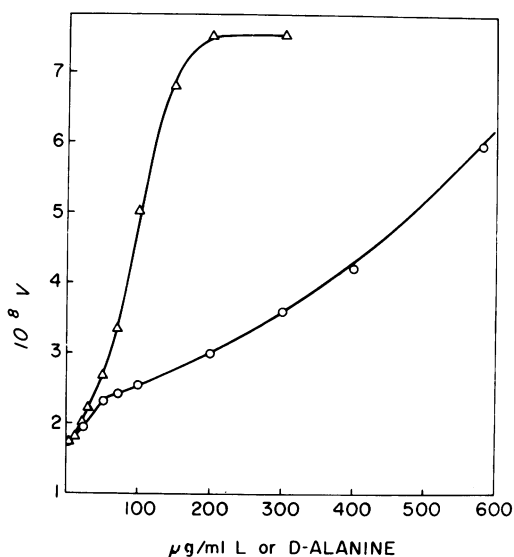

FIG. 5. Suppression of the cell division abnormality of MAD-1 by D-alanine and L-alanine. MAD-1 was grown on glucose-minimal medium containing the concentration of D- (△) and L-(○) alanine shown for about four generations; then, V was calculated as described in Materials and Methods. V for CR34 under all of these conditions is about 7.5 × 10⁸.

the V_{max} of L-alanine racemase from sonically treated preparations of both mutant and wild-type cells grown at 30 or 40 C in the presence of permissive or nonpermissive carbon sources, respectively. There was no difference in the K_m for L-alanine (2.7 mM) or the V_{max} for L-alanine racemase between the mutant and its parent CR34 under any of the conditions examined.

We also analyzed D-alanine-D-alanine synthetase, the next enzyme in the pathway of incorporation of D-alanine into the cell wall. It was thought that a change in the K_m of this enzyme for D-alanine might require higher concentrations of D-alanine in order to effect proper cell wall biosynthesis. However, no difference in the K_m (25 mM) and the V_{max} for D-alanine-D-alanine synthetase was found between the mutant and the wild-type strains.

The transport of D-alanine was studied by the method of Wargel et al. (11). At a D-alanine concentration of 2 × 10⁻⁵ M, the initial rate of uptake was 6 × 10⁻⁴ μmole/min/mg of protein for both MAD-1 and CR34, in agreement with the value previously found for E. coli K-12 (11).

Nature of the phenotypic suppression by D-alanine. As described in the accompanying paper, MAD-1 was selected because of an in-

creased permeability to lactose. In addition, it had other permeability changes which made it more sensitive to deoxycholate and to actinomycin D than was the wild-type strain. It was thus of interest to see whether D-alanine, which caused a change of the morphology to normal, would also suppress the permeability increase which was evidenced by increased sensitivity to certain chemical agents. Figure 6 illustrates such an experiment. Part A shows the effect of actinomycin D and deoxycholate on the parental strain, CR34. There is only a slight inhibition of growth by 25 μg of actinomycin D per ml, and the generation time is halved by 0.2% deoxycholate. In Fig. 6B, however, we see the response of MAD-1 to these agents, in which there is complete inhibition of

growth by the same concentration of actinomycin D and lysis of the cells by deoxycholate. When MAD-1 is grown in the presence of D-alanine (Fig. 6C), its sensitivity to actinomycin and to deoxycholate are much decreased, approaching that of the wild-type strain. Therefore, D-alanine is able to reverse the increased permeability of MAD-1 as well as its morphologic and cell division abnormality. Another experiment was also designed to test for a cell surface modification and whether or not it was corrected by D-alanine (Fig. 7). Resting cells of MAD-1 are exquisitely sensitive to lysozyme, even in the absence of ethylenediaminetetraacetate, and to sodium deoxycholate. Thus, one can measure lysis of the cell preparation by following the decrease of optical density at 600 nm. Figure 7A shows the effect of low concentrations of lysozyme (2.5 μg/ml) on MAD-1 cells which had been previously grown
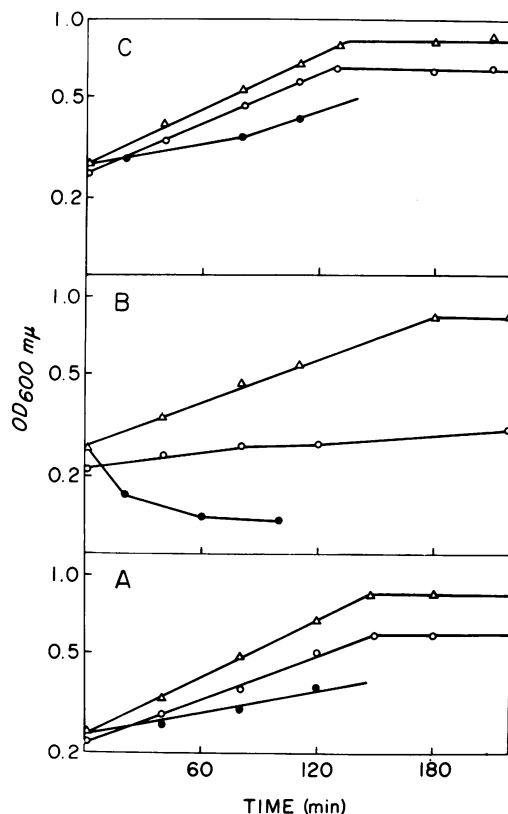

FIG. 6. Suppression of the MAD-1 permeability alteration by growth on D-alanine. Bacteria were grown at 40 C in glucose-minimal medium to the mid-exponential phase and then inoculated into the same medium containing: (△) no additions; (O) actinomycin D, 25 μg/ml; (●) sodium deoxycholate, 2 mg/ml. Part A shows the parent strain, CR34; B shows MAD-1; C is MAD-1 which had been pregrown on medium containing 200 μg of D-alanine per ml and inoculated into the same medium for this experiment.
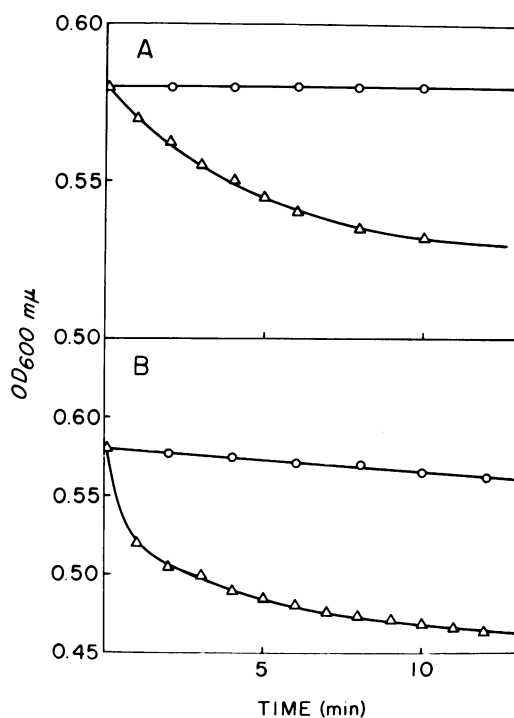

FIG. 7. Effect of D-alanine on suppressing the lysis of MAD-1 by lysozyme and deoxycholate. MAD-1 was grown into the logarithmic phase at 40 C in glucose-minimal medium with (O) or without (△) supplementation by D-alanine (200 μg/ml). One milliliter of culture was transferred to the thermostatted compartment (40 C) of a Gilford spectrophotometer and, at zero time, (A) lysozyme (final concentration 2.5 μg/ml) or (B) sodium deoxycholate (final concentration 2.5 μg/ml) was added. The optical density of the suspensions was then monitored.

in glucose-minimal medium, either with or without supplementation by D-alanine. It is clear that cells which have grown with D-alanine supplementation are more resistant to this low concentration of lysozyme than are those grown in the absence of D-alanine. The cells which have been phenotypically suppressed by D-alanine behave as do wild-type cells under these circumstances. Figure 7B shows a similar experiment performed with a low concentration of sodium deoxycholate, with essentially the same results. Therefore, these results lend further support to the idea that D-alanine supplementation corrects both the increased permeability and the morphological and cell division abnormality of MAD-1.

Evidence that the defect in MAD-1 is due to an increase in autolysis rather than a decrease in cell wall biosynthesis. Although it appeared from our results to this point that MAD-1 had an alteration in its cell wall composition which could be corrected by a high concentration of D-alanine, there was still some question as to whether the defect leading to the cell wall alteration was caused by a decrease in the rate of biosynthesis of cell wall, an alteration in the structure of the cell wall, or an increase in the rate of its autolysis. To investigate this, we examined the effect of penicillin G and D-cycloserine, two antibacterial agents known to inhibit cell wall biosynthesis. MAD-1 growing on a nonpermissive carbon source is more susceptible to inhibition by penicillin G (50 units/ml) or D-cycloserine (2×10^{-6} M), in that both of these agents lead to an immediate inhibition of cell growth with MAD-1, whereas, with the parental strain, there is a pronounced lag before they had any effect (Fig. 8).

This result is consistent with an alteration in the cell wall of MAD-1, but it was not clear whether this is caused by an altered rate of synthesis or lysis, or by an alteration in the cell wall structure. We decided to inhibit cell wall biosynthesis completely in affected and suppressed cultures of MAD-1 and ask whether suppression by D-alanine would protect the cells from autolysis. If the defect in MAD-1 was really an increased autolysis, caused either by an alteration in cell wall structure or an excessively active lytic enzyme, one might predict that even phenotypically suppressed cells, pregrown with D-alanine, would be more readily lysed upon inhibition of cell wall biosynthesis than is the wild-type strain.

The results of an experiment to test this hypothesis are shown in Fig. 9, where the
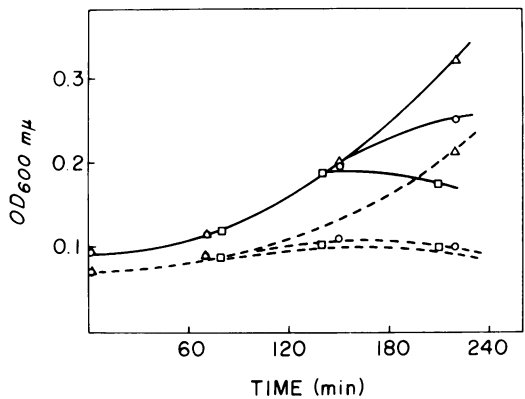

FIG. 8. *Effect of penicillin G or D-cycloserine on the growth of MAD-1 (---) or CR34 (——). Cells growing logarithmically on glucose-minimal medium at 30 C were supplemented with 50 units of penicillin G per ml (O), with 2×10^{-6} M D-cycloserine (□), or with no addition (Δ) at zero time, and further growth was followed by optical density.*
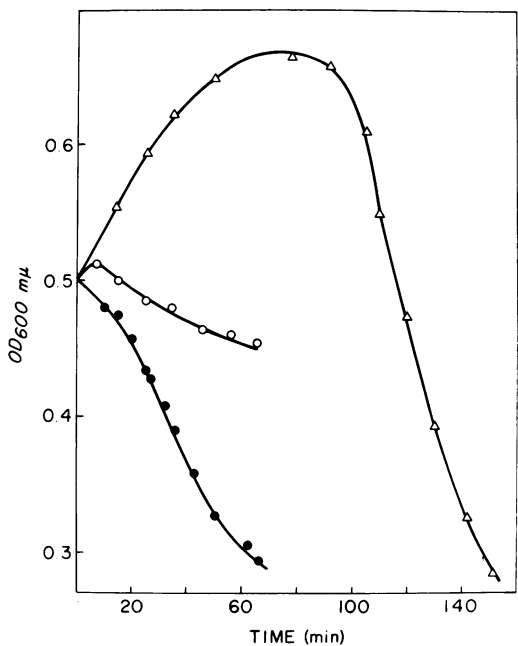

FIG. 9. *Effect of inhibition of cell wall biosynthesis on lysis of MAD-1 and CR34. Cells were growing logarithmically at 40 C in glucose-minimal medium and then transferred in 1-ml samples to the thermostatted compartment (40 C) of the Gilford spectrophotometer, in the presence of penicillin G (50 units/ml). The optical density change of CR34 (Δ), MAD-1 (●), and MAD-1 suppressed by D-alanine (O) was measured. D-Alanine had no effect on CR34.*

change in optical density of a suspension of parental cells or MAD-1 cells pregrown in the presence or absence of D-alanine is indicated. The parent, CR34, continues to increase in optical density for over 1 hr in the presence of 200 μg of penicillin G per ml. The mutant MAD-1 lyses immediately when placed in the presence of penicillin G. Even when the mutant is pregrown on D-alanine, there is still a rather marked inhibition of growth followed by premature lysis. A similar result is seen if D-cycloserine (2×10^{-6} M) is used to inhibit cell wall biosynthesis. Since D-cycloserine is transported by the same system as D-alanine (11), and D-alanine transport is not altered in MAD-1 (see above), the premature lysis in the presence of D-cycloserine (and, by analogy, penicillin) is probably not due to a marked increase in the permeability of the cells to these agents. The premature lysis of MAD-1 would be expected if the defect in MAD-1 resulted in an increase in the autolytic activity of that strain, rather than a defect in the rate of cell wall biosynthesis.

Peptidoglycan structure and synthesis in MAD-1. To pursue the nature of the defect in MAD-1, we examined the rate of biosynthesis and the structure of peptidoglycan from this strain, compared to its wild-type parent. For these studies, highly purified sacculi were obtained by the procedure of Schwarz et al. (10), which involves boiling the particulate fraction of *E. coli* in sodium dodecyl sulfate, followed by multiple washings to remove protein contaminants. Sacculi from MAD-1 retained the pleiomorphic properties of the cells, as ex-

TABLE 3. *Incorporation of radioactive precursors into sacculi of MAD-1 and CR34[a]*

Strains	Amt of incorporation of precursor (counts per min per mg of sacculus)		
	DAP	D-Alanine	L-Alanine
CR34	42,700	6,836	10,900
MAD-1	43,200	10,155	9,052

[a] Cells were grown at 40 C on glucose-minimal medium until the mid-exponential phase, and then radioactive precursor was added for an additional 60 min. The reaction was stopped by transferring the cells to an ice bath, and sacculi were prepared by the boiling sodium dodecyl sulfate technique of Schwarz et al. (10). The quantity of sacculi was estimated from the Lowry reaction. Radioactive precursors were: ^{3}H-diaminopimelic acid (DAP), 5 μCi/ml, 5 μg/ml; ^{14}C-D-alanine 2 μCi/ml, 200 μg/ml; ^{3}H-L-alanine, 1 μCi/ml, 5 μg/ml. Incorporation was in the presence of chloramphenicol (200 μg/ml) after a 15-min preincubation with the antibiotic.

pected from previous observations (10). The incorporation of radioactive cell wall precursors into such fragments is shown in Table 3. There is no difference between the mutant and the wild type in the incorporation of L-alanine or diaminopimelic acid, whereas the mutant has incorporated 1.5 times more D-alanine than the wild type in the 60 min of incubation shown. The data of Table 3 are for 60 min of incorporation; the same relative results are seen when initial rates of incorporation into cell wall are measured. In the latter instance, for which the data is not shown, the initial rate of incorporation of the three cell wall precursors into CR34 was about 2 nmoles/min/mg of cell protein. A similar rate was found for L-alanine and diaminopimelic acid incorporation into MAD-1, but the rate of incorporation of D-alanine was 1.5 times higher. Therefore, these experiments show that there is no marked defect in cell wall biosynthetic capacity in MAD-1, at least for the step which is rate-limiting under our conditions of measurement.

Sacculi preparations from MAD-1 and CR34 were then examined for the quantities of peptidoglycan fragments seen after lysozyme digestion (8, 12). There is no difference in the ninhydrin reactivity of the fragments obtained, indicating that there is no marked alteration in peptidoglycan composition (Fig. 10). The radioactivity of the major spots on the chromatogram was measured by elution and scintillation counting, and the incorporation of D-alanine and diaminopimelic acid into each spot was no different from the ratios seen on the whole saccular preparation (Table 3). Thus, the gross compositions of the peptidoglycans from CR34 and MAD-1 seem to be identical despite the bizarre form of the peptidoglycan sacculus from the mutant cells.

DISCUSSION

The defect in cellular division and morphology in MAD-1 is expressed only on minimal medium with specific carbon and energy sources, and this defect can be reversed by supplementation with D-alanine. It is not yet clear why the defect is conditional upon the carbon source used for growth. We have assayed hexokinase, but have not exhaustively analyzed all of the enzymes in the metabolism of the various permissive and nonpermissive sugars. Nonetheless, the classes of sugars which act as permissive or nonpermissive agents do not provide an obvious suggestion for the site of the defect. Perhaps the expression of the defect is dependent upon the steady-state level of pyruvate generated by
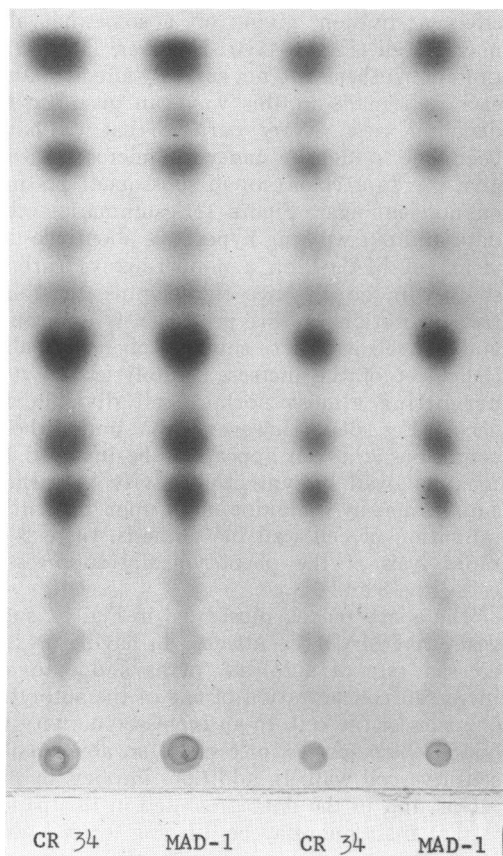
FIG. 10. A chromatogram of lysozyme-digested sacculi from CR34, and MAD-1. Sacculi were prepared as in Table 3 and digested with lysozyme by the procedure of Schwarz and his collaborators (8, 10). Chromatographic separation was on cellulose thin-layer plates (Eastman) in a solvent consisting of butanol-pyridine-acetic acid-water (300:201:60 240). This solvent was more convenient than using the upper phase of butanol-acetic acid-water as previously described (10), but gave essentially the same distribution of chromatographic spots. The separations on the left were of 5 µg of saccular digestion products; those on the right, of 2 µg.

growth on these different carbon sources, since pyruvate is a precursor for cell wall biosynthesis. It is conceivable that the steady-state level of D-alanine is regulated by the steady-state level of pyruvate. Alternatively, the effect we are seeing might be some kind of catabolite repression, although cyclic AMP does not effect a reversal of the morphological abnormality. That the abnormality may be related to a defect in sugar transport was suggested as a possibility from some earlier work on a sugar transport mutant of E. coli K-12 (1)

which showed a round cell morphology. This possibility was obviated by the data in Fig. 2, which show that there is no significant defect in transport of the carbon sources used for growth of MAD-1. At present, we do not understand the relationship between the carbon source used for growth and the morphologic abnormality.

On the other hand, it is rather clear from our studies that both the increased permeability, which was used as a selection technique, and the morphologic abnormality may be corrected by D-alanine. This suggests that one mutation is responsible for the several defects and could be explained by the following considerations (Fig. 11). Both the morphology of the cell and the permeability barrier may be related to the integrity of the peptidoglycan. Thus, an alteration of peptidoglycan which leads to an altered cell morphology might also lead to an increase in cellular permeability, if peptidoglycan constituted a portion of the permeability barrier, or if the membrane is distorted under these conditions.

In attempting to analyze the nature of the defect which can be suppressed by D-alanine, we have found that there is no dramatic inhibition of cell wall biosynthesis in MAD-1, nor is the peptidoglycan composition markedly altered. D-Alanine is preferentially incorporated into the cell wall of MAD-1 cells growing under nonpermissive conditions (on glucose); this might reflect a decrease in pool size or an excess of acceptor sites for D-alanine in the cell wall. Our measurements of peptidoglycan fragments showed no support for the latter possibility, but perhaps such sites would not have been detected by our technique. The data from Fig. 8 and 9 suggest that the primary defect in MAD-1 might be in autolysis, with the mutant having an abnormally high or uncontrolled autolytic activity; D-alanine could suppress the defect by inhibiting such an autolysin. We are presently investigating the several autolytic activities of CR34 and MAD-1, in order to see whether such a defect accounts for the abnormality in MAD-1.

Our results are compatible with the hypothesis that there is an increase in autolytic activity which is not compensated for by increased biosynthesis. As a result of this relative deficiency in biosynthesis, which is really an inability to keep up with the autolytic process, the cell bulges out in the area where the cell wall is deficient (Fig. 1B). Lysis, however, does not apparently occur to any great extent, since the amount of free β-galactosidase in the medium is less than 1% of the total in the
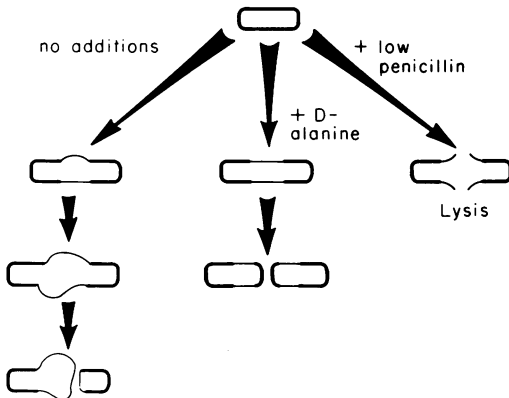
A. WILD TYPE
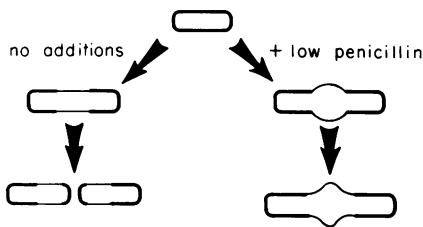
B. MAD-1

FIG. 11. *A model for the growth of MAD-1 and wild-type E. coli. Normal growth of wild-type E. coli involves autolysis of the cell wall (4) in order to form daughter cells. Low concentrations of penicillin induce filament formation by inhibiting cell division, perhaps by causing autolysis at a rate which prohibits cross septum formation (10). These phenomena are shown in an exaggerated fashion in 10A, where a thinning of the cell wall suggests autolytic activity involved in normal cell division, whereas a bulge of the thin section indicates a potential imbalance toward autolysis during treatment with low concentrations of penicillin. In B, without any addition to a nonpermissive medium, MAD-1 actually does bulge and makes large abnormal cells. The autolytic process is normally paced with cell division if D-alanine is used to suppress the abnormality. Low penicillin concentrations push the fragile equilibrium in MAD-1 too far towards autolysis, and the cells burst.*

cells. Under these conditions, cell wall biosynthetic activity is directed towards "patching up" the cell wall by directing the precursors, which might be used in the division event, into a repair process. Thus, the cells do not divide but instead become larger. We therefore view the process of accelerated lysis and secondarily altered biosynthesis as leading to a pleimorphic *E. coli* cell which only eventually can

effect a division, giving off a somewhat abnormal cell (Figure 11B). However, DNA and protein synthesis are not severely affected, and each cell made in this way can give rise to daughter cells. Every particle that we have been able to identify under the microscope can give rise to a colony upon subsequent plating on nutrient agar. Figure 11B summarizes the data and our working hypothesis about the division of MAD-1 on a nonpermissive carbon source. In the presence of D-alanine, the relative limitation of this precursor is overcome and the cell is able to suppress phenotypically the effect of the increased autolytic activity, permitting almost normal cell division to occur (Fig. 6C). However, even under these conditions, the cell appears to be involved in maximal wall biosynthetic activity to permit suppression by D-alanine, and, upon penicillin inhibition of cell wall biosynthesis, there is a rapid lysis of the phenotypically suppressed cells (Fig. 8 and 9).

The above model, illustrated in Fig. 11, suggests that MAD-1 is affected in having an increased rate of autolysis, perhaps due to an increased concentration of one of the autolytic enzymes of the cell, to an increased activity of one of these species, or even to an abnormally sensitive cell wall. In addition, implicit in the model and in the data presented in this paper is that D-alanine may be limiting for cell wall biosynthesis under certain growth conditions. In wild-type cells this limitation would not lead to a serious derangement in growth or division, because the autolytic activity is well paced with the cell division cycle. In MAD-1, however, where there apparently is increased autolytic activity, the limiting nature of the D-alanine supply could become manifest. Low concentrations of penicillin G serve to induce filament formation in the parent, CR34, probably because of partial inhibition of cell wall biosynthesis (10). Under these conditions, there is lysis of MAD-1, which is consistent with the presence of higher levels of autolytic activity in this mutant, even when it is suppressed in the presence of D-alanine (Fig. 9). We have not yet found any significant difference in the peptidoglycan fragments from lysozyme-hydrolyzed cell wall preparations, suggesting that the defect is not a qualitative one in the structure of the cell wall, but rather an increase in the level or activity of an autolytic enzyme. We are continuing to investigate the relationship between the apparent increase in autolysis in MAD-1 and the cell division cycle of *E. coli.*

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