The Nutritional Requirements of Methicillin-dependent and -resistant Strains of *Pediococcus cerevisiae*

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SUMMARY

A new methicillin-dependent and -resistant substrain (called MRD) of *Pediococcus cerevisiae* was developed by serial passage followed by replica-plating. Other methicillin-resistant, but not -dependent, substrains were isolated after treatment of the same parent strain with a mutagen. A methicillin-independent partial revertant, still resistant to the drug, was isolated from the original methicillin-dependent and -resistant substrain (CRD) developed several years ago.

The requirements of some of these strains for acetate, vitamins and amino acids were compared. All except the parent methicillin-sensitive strain required pantothenate for growth, but no other consistent differences were found. The parent, but not strain CRD, grew without lysine added to the medium, though 19 other amino acids were needed by each strain. Both of these strains fermented glucose to lactate (mainly the L-isomer) in the absence or presence of methicillin.

INTRODUCTION

A substrain of *Pediococcus cerevisiae* ATCC8081 that was dependent on methicillin for growth in a chemically partly defined medium was isolated by White (1968*a*). This substrain (called *P. cerevisiae* CRD) was 40 times more resistant to methicillin than the parent strain, but showed little increased resistance to benzyl penicillin. There were other differences between strain CRD and the parent in their nutritional requirements for tetrahydropteroyl-glutamate and acetate.

To determine whether any of these differences were directly attributable to methicillin dependence, a new methicillin-dependent resistant strain, a partial revertant (i.e. no longer methicillin-dependent, but still resistant) strain of CRD, and new methicillin-resistant but not dependent strains were isolated for comparative purposes. This second isolation of a methicillin-dependent strain suggested that the production of such strains is not the result of a very rare genetic event.

Markov & Saev (1956) reported that with some benzyl penicillin-resistant strains of *Staphylococcus aureus* (the parent strain of which required thiamin and nicotinic acid), the growth-promoting effect of thiamin could be reproduced by benzyl penicillin at very low concentrations. An intact β -lactam ring was not necessary; penicilloic acid gave a similar effect. A substance was postulated to arise from benzyl penicillin that acted in a similar manner to thiamin (Markov & Saev, 1957). Although these experiments were not entirely convincing, they prompted a comparison of the vitamin requirements of *P. cerevisiae* 8081 and the various substrains. The amino acid requirements of the parent and CRD strains, and

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the abilities of these two strains to ferment glucose in the presence and absence of methicillin were also studied.

A preliminary account of part of this work has been given (Widdowson & White, 1971).

METHODS

Organisms. *Pediococcus cerevisiae* ATCC8081 and CRD (the original methicillin-dependent substrain) were maintained as described previously (White, 1968*a*). The isolation of other substrains is described below.

Media. The partly defined medium (containing leucovorin, the calcium salt of 5-formyl tetrahydropteroylglutamate) was that described by White & Nichol (1963) and will be referred to as medium SD. Peptone+yeast extract+glucose medium (PYG) and PYG+acetate (PYGAc) were as described by Wilkinson & White (1973), except that a salts solution (10 ml/l) was added; this contained (in 500 ml of solution in distilled water): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 g; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 2 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g; NaCl, 1 g; concentrated (11.6 M) HCl, 0.5 ml. Media were autoclaved at 121 °C for 15 min. Solutions of methicillin and benzyl penicillin were Seitz filtered and added to the cooled, sterile media.

To grow surface colonies of these microaerophilic organisms, sodium thioglycollate (375 µg/ml) was added to media, which also contained 1 % (w/v) agar. Solutions of sodium thioglycollate were sterilized by Seitz-filtering, and mixed into autoclaved medium at 44 °C.

Assessment of growth. All plates and liquid cultures (in stationary flasks or tubes) were incubated at 37 °C and turbidities were measured in an EEL photoelectric colorimeter as described by White (1968*a*).

Isolation of new methicillin-dependent and -resistant strains. (i) Methicillin-dependent resistant strain. *Pediococcus cerevisiae* 8081 was passaged in the presence of methicillin as described by White (1968*a*). After 31 transfers the culture was tested for the presence of organisms unable to grow without methicillin by plating on to medium SD containing methicillin (50 µg/ml), and then replicating (Lederberg & Lederberg, 1952) the resulting colonies (after incubation for 2 days) on to media with and without methicillin. Colonies that grew only in the presence of the drug were picked for further testing.

(ii) Methicillin-independent revertant strains from CRD. Large inocula (0.1 ml of washed, exponential-phase organisms, equivalent to 0.3 to 0.03 mg dry wt/ml) were plated on to medium SD without methicillin. Any large colonies that grew within 50 to 60 h were picked off and tested for growth without methicillin on fresh plates.

(iii) Methicillin-resistant strains. Washed, exponential-phase parent organisms (equivalent to about 0.5 to 0.7 mg dry wt/ml) were incubated at 37 °C for 30 min in 0.1 M-tris – maleate buffer, pH 6.0, containing the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; 100 µg/ml). The organisms were then washed twice in sterile water and inoculated into liquid PYG medium to allow expression of possible mutations. Medium SD (20 ml), containing methicillin at various concentrations, was inoculated at 44 °C with 1 ml PYG culture and then poured into a plate and allowed to set. A 10 ml overlay of the same uninoculated medium was added to provide conditions for microaerophilic growth (White, 1968*a*). This procedure was used before additions of thioglycollate had been introduced. Colonies of possible methicillin-resistant organisms were picked out with a straight wire and retested by stabbing into fresh methicillin-containing plates.

(iv) Methicillin-sensitive but -dependent strains. Exponential-phase parent organisms were treated with NTG as before, and the washed organisms were incubated in medium

SD for 8 h at 37 °C to allow growth of methicillin-independent bacteria. Sterile D-cycloserine was then added to a final concentration of 1 mg/ml and incubation was continued for a further 18 h. The organisms were washed twice and an inoculum equivalent to 0.5 mg dry wt was used to inoculate 10 ml of medium SD containing methicillin (5 µg/ml), which was then incubated overnight. After a further two cycles of treatment with cycloserine, the organisms were washed twice, diluted and tested for methicillin dependence by replica-plating on to medium SD with and without methicillin (5 µg/ml).

Determination of sensitivities to penicillins. Organisms were grown into exponential phase in PYG medium, washed twice and 0.1 ml of a suspension (equivalent to about 3 µg dry wt/ml of water) was used to inoculate 2.4 ml of medium SD in each of a series of 19 × 150 mm test tubes containing either methicillin (0 to 600 µg/ml, in steps of 25 µg/ml) or benzyl penicillin (0 to 5.0 µg/ml, in steps of 0.5 µg/ml). After incubation for 3 days the resistance of the organism was taken to be the highest concentration of the antibiotic which permitted growth.

Vitamin requirements. Exponential-phase organisms (grown in medium SD, containing 50 µg methicillin/ml where necessary) were washed once in sterile water. Samples of the washed bacteria (equivalent to 0.6 µg dry wt/ml) were used to inoculate a series of 19 × 150 mm tubes of medium SD (5 ml final volume) from which single vitamins were omitted. Medium was prepared without vitamins and sterilized by Seitz-filtering. Vitamins were added (to the same final concentration as in the complete medium) as separate solutions that had been sterilized by filtration.

Amino acid requirements. Medium SD was prepared without the amino acids plus acid-hydrolysed casein usually added. These were replaced by the mixture of amino acids used in the fully defined medium of Sauberlich & Baumann (1948). To allow the amino acid content to be varied, each amino acid was made up in concentrated solution so that the addition of 0.1 ml of each gave the final concentration required in 5 ml of medium. In a series of 19 × 150 mm tubes each amino acid was omitted in turn from the complete mixture which supported growth of *P. cerevisiae* 8081. Methicillin (50 µg/ml) was added to medium for strain CRD. Tubes were inoculated as in the study of vitamin requirements.

Fermentation of glucose. Strains 8081 and CRD were grown for 48 h (into stationary phase) in PYGA_c and PYGA_c + methicillin (100 µg/ml), respectively, in 2 l flasks containing 400 ml medium. Each flask was inoculated with 1 ml of the appropriate overnight culture in medium SD (plus methicillin for CRD). The bacteria were harvested by centrifuging, washed twice in 0.9 % NaCl and resuspended in water to about 30 mg dry wt/ml. Each suspension (0.5 ml) was incubated at 37 °C in Warburg flasks containing sodium bicarbonate solution (20 µmol in 2.5 ml total volume after all additions) under 5 % (v/v) CO₂ in N₂. After tipping in glucose (5 µmol) the evolution of gas was followed manometrically (Umbreit, Burris & Stauffer, 1964). Acid was tipped from a second side arm when the first gas evolution was over. The flask contents were assayed for glucose with glucose oxidase (Bergmeyer & Bernt, 1965), for total lactate (Barker, 1957) and for L-lactate (Hohorst, 1965). The experiment was repeated for both strains with methicillin (100 µg/ml) in the Warburg flasks, and again repeated with phosphate buffer pH 6.5 (20 µmol/2.5 ml, replacing bicarbonate) under N₂.

Chemicals. Lipoic acid was a gift from Dr J. R. Guest (University of Sheffield). Other chemicals were commercially available (and of analytical reagent purity where possible), or their origins were given by White (1968a)

RESULTS

Production of a new methicillin-dependent strain

After 31 passages with methicillin, the resistance of *P. cerevisiae* to the antibiotic was increased about 50-fold. Cultures grew in medium without methicillin as quickly as in medium containing it until passage 20, when, after incubation for 17 h, the tubes containing 100 µg methicillin/ml showed distinct growth whereas there was only faint turbidity in drug-free medium; after incubation for a further 23 h, however, growth had become of equal density in both tubes. During the following 11 passages differences in the growth of the cultures plus and minus methicillin were not consistent. Growth in medium without drug was slower in passages 23, 24, 26 and 30. These inconsistencies might have been due to the presence in the culture of a mixture of methicillin-dependent and -independent organisms, the latter being revertants to independence or organisms that had never become dependent.

Methicillin-dependent organisms were isolated after passage 31 by replica-plating. No replication was attempted during the earlier passages. Sodium thioglycollate in the solid medium enabled colonies to grow on the surface of plates, and did not inhibit the rate of growth or decrease the number of colonies formed in comparison with colonies grown by the uninoculated-overlay technique (White, 1968*a*). Methicillin-dependent organisms were present in the culture from passage 31 in a proportion of about 1 colony in 20. After suspected methicillin-dependent colonies had been tested on solid medium SD with and without methicillin (50 µg/ml), a methicillin-dependent colony was selected and designated as strain MRD (methicillin-resistant-dependent).

Cultures of strain MRD in liquid medium SD became turbid as rapidly in the absence of drug as in its presence, which suggested a high rate of reversion to drug independence. Organisms were plated onto medium plus and minus methicillin, and colonies that had grown after 3 days were counted. Approximately 1 colony in 15 grew in the absence of methicillin. This suggests that the revertants might grow faster in the absence of the drug than MRD did in its presence.

Methicillin-resistant strains

Strain 8081 Met^{Ra} was produced by NTG treatment of strain 8081. It was inhibited by concentrations of methicillin higher than 75 µg/ml. Strain Met^{Ra} was subjected to a further cycle of NTG treatment, and a strain (called Met^{Rb1}) was isolated which could grow in the presence of 150 µg methicillin/ml. Another strain (Met^{Rb2}) was obtained from a spontaneous mutation of Met^{Ra} by plating a large inoculum onto medium SD containing 150 µg methicillin/ml and selecting a single colony which grew.

Revertants of strain CRD to methicillin independence occur at a frequency of 1 in 10⁵ (White, 1968*a*). A revertant colony was picked from drug-free medium SD, and was called strain CR since it retained methicillin resistance (see below).

Methicillin-sensitive yet -dependent organisms

No success was achieved in six attempts (see Methods) to isolate organisms of this class. Such organisms would have needed to be able to use methicillin as a growth factor at concentrations of 5 µg/ml or less.

Benzyl penicillin-resistant strain

The method was identical to that used for isolating methicillin-dependent organisms, except that benzyl penicillin replaced methicillin. Earlier attempts to develop penicillin

Table 1. *Inhibitory concentrations of methicillin and benzyl penicillin for P. cerevisiae 8081 and substrains*

Washed, exponential-phase organisms (grown in PYG) were diluted to an estimated colorimeter reading of 0.01 (about 3 μ g dry wt/ml). Tubes of medium SD (2.4 ml) containing various concentrations of the antibiotics were inoculated each with 0.1 ml of suspension and incubated at 37 °C.

Strain	Inhibitory concentration (μ g/ml)*	
	Methicillin	Benzyl penicillin
8081	10	1.0
CRD	500	1.0†
MRD	400	2.0†
CR	300	1.5
Met ^{Ra}	75	2.0
Met ^{Rb1}	300	1.0
Met ^{Rb2}	300	2.0
Pen ^R	300	6.0

* Maximum concentrations in which growth occurred after 3 days.

† Determined with methicillin (50 μ g/ml) in the medium.

resistance in this way had been unsuccessful (White, 1968*a*) but no special difficulty was found in the present experiments. Resistance increased by small steps during passages 1 to 23, from a maximum tolerated dose of 1 μ g/ml up to 8 μ g/ml. No further increase in resistance occurred; indeed, after passage 31 only a 6-fold gain in resistance had been achieved. Organisms from this culture were designated 8081 pen^R. At no stage did growth appear sooner when penicillin was present in the medium than in its absence. Growth without benzyl penicillin was almost always faster, and the final turbidity attained was often greater in drug-free medium. Whether pen^R reverts to sensitivity, or whether the organisms lyse in the presence of higher concentrations of penicillin, has not been established. No attempts were made to isolate benzyl penicillin-dependent colonies by replica-plating.

Properties of the parent and substrains compared

Resistance to methicillin and benzyl penicillin. The inhibitory concentrations of benzyl penicillin and methicillin were determined for each strain (Table 1). Large increases in methicillin resistance did not lead to comparable increases in resistance to benzyl penicillin, although the benzyl penicillin-resistant strain 8081 pen^R showed a 15- to 30-fold increase (depending on the period of incubation) in methicillin resistance. The concentration of methicillin (50 μ g/ml) added to the medium for estimation of the benzyl penicillin resistance of CRD and MRD was the highest that could be used without decreasing the resistance to benzyl penicillin. Strain CR, although no longer dependent on methicillin, still showed resistance to this antibiotic.

Thymidine requirement. White (1968*a*) found that neither parent nor CRD organisms grew in medium SD if leucovorin were omitted, even if pteroylglutamate (and methicillin) were added. However, the parent strain could grow if leucovorin was replaced by thymidine (0.1 mg/l), though to only one-third of the final turbidity achieved with leucovorin. Strain CRD did not grow in such medium (plus methicillin), even after incubation for 120 h.

To help determine whether this difference was in any way related to the biochemical mechanism of dependence on methicillin, growth of the newly-developed strains was examined on solid medium, to assess whether ability to grow was a property of the whole

Table 2. *Vitamin requirements of P. cerevisiae* 8081 and substrains

Organisms were harvested from medium SD (plus methicillin if needed for growth), washed in water and diluted to an estimated colorimeter reading of 0.002 (about 0.6 µg dry wt organisms/ml). These suspensions (0.1 ml) were used to inoculate 4.9 ml portions of medium SD from which the vitamins were omitted singly.

Vitamin omitted	Strain						
	8081	CRD*	MRD*§	CR	Met ^{Ra}	Met ^{Rb1}	Met ^{Rb2}
Leucovorin	—	—	—	—	—	—	—
Leucovorin (replaced by thymidine)	+++	—	+++	++	+	—	+++
Pantothenate	+++	—	—	—	—	ND	ND
Biotin	±	—	±	ND	±	ND	ND
Nicotinic acid	±	—	—	ND	—	ND	ND
Riboflavin	+++	++	+++	ND	+++	ND	ND
Thiamin	+++	++	+++	ND	+++	ND	ND
B ₆ †	+++	++	+++	ND	+++	ND	ND

+++ , Heavy growth; —, no growth; ND, not determined.

* Methicillin (50 µg/ml) added.

† Pyridoxin + pyridoxal + pyridoxamine all omitted together.

§ These liquid cultures of strain MRD would have contained revertants to drug independence, even though methicillin was present. Consequently, the ability to grow in the absence of a given vitamin is not necessarily a property of strain MRD. A separate experiment (see text) confirmed that strain MRD could grow with thymidine in place of leucovorin.

population or of only a few colonies. Strains MRD, 8081 Met^{Rb2} and CR were all able to grow with thymidine in place of leucovorin, whereas 8081 Met^{Ra} grew only poorly and 8081 Met^{Rb1} did not grow with thymidine. Thus, the inability to grow on a medium in which leucovorin is replaced by thymidine is not a property that is essentially linked to methicillin dependence.

Vitamin requirements. Strains 8081 (parent), CRD, MRD, CR and 8081 Met^{Ra} were investigated (Table 2). All the strains that were resistant to methicillin showed a requirement for pantothenate, which was not required by the parent. None of the resistant strains grew if medium without pantothenate was supplemented with β-alanine (1 µg/ml) plus pantolactone (1 µg/ml). Medium SD (in which all the substrains were isolated) contained sodium pantothenate (1 mg/l).

Amino acid requirements. The parent strain grew in the chemically defined medium that was produced by replacing the acid-hydrolysed casein in medium SD by amino acids. Strain CRD required methicillin (about 20 µg/ml was optimal) for growth in this medium, but only light turbidity was produced even after incubation for 2 days or longer. Strain 8081 grew in the absence of aspartic acid or asparagine (but not of both) or of lysine; omission of any other amino acid prevented growth. Strain CRD did not grow when any single amino acid was omitted.

Acetate requirement. Strain CRD grew without methicillin in medium SD containing decreased concentrations of sodium acetate (White, 1968a). The inhibitory effect of 2 % (w/v) sodium acetate might have been a consequence of autoclaving, because the medium with acetate became browner on autoclaving than did medium without acetate. Medium SD lacking acetate was therefore made, without any heating during its preparation, and was sterilized by filtering and dispensed into sterile tubes. Various amounts of sodium acetate were added, as a solution adjusted to pH 6.5 and sterilized by filtering. Tubes were inoculated with 8081 or CRD (overnight cultures diluted to the equivalent of a colorimeter reading of 0.002). Uninoculated medium was also incubated, with and without methicillin, to check that none of the solutions were contaminated.

Table 3. *Ability of P. cerevisiae* 8081 and methicillin-resistant substrains to grow in the absence of acetate

Organisms were grown overnight in medium SD, plus methicillin (50 µg/ml), for all strains except the parent. Inocula (0.1 ml) of about 1000 colony-forming units from each culture were plated on medium SD plus thioglycollate but lacking sodium acetate. Plates were incubated at 37 °C for 72 h. The number of colonies (where these appeared) was similar in all cases.

Strain	Growth on medium without acetate	
	– Methicillin	+ Methicillin (50 µg/ml)
8081	+++	–
CRD	–	–
CR	±	–
MRD	–	+++
Met ^{Ra}	++	++
Met ^{Rb1}	+++	+++
Met ^{Rb2}	+++	+++

+++ , Large colonies; ± , very small colonies; – , no growth.

The inhibitory effect of acetate was clearly not a result of autoclaving. Strain CRD was inhibited by 2 % (w/v) sodium acetate unless methicillin was present, and 4 % was inhibitory even when the drug was added. Growth without methicillin occurred with 0.1 to 1 % acetate; 0.4 % was optimal. The parent strain was not inhibited by 4 % (w/v) acetate and could grow, to a limited extent, in the absence of acetate. Addition of lipoic acid in the range 0.1 to 100 µg/l did not improve the growth of either strain in filtered medium without acetate. The newly-isolated strains were tested on solid medium SD without acetate (Table 3). All strains, except CRD or CR, grew without acetate. Thus, the requirement for acetate shown by strain CRD is not a property that is necessarily linked to methicillin-dependence.

Fermentation of glucose. The concentration of glucose in medium SD could be decreased from 25 to 10 g/l before there was a decrease in the final yield of the parent strain. Strain CRD grew less heavily than the parent strain in medium SD (plus 100 µg methicillin/ml) and there was little change in the yield of bacteria until the concentration of glucose was less than 5 g/l. With none of the altered concentrations of glucose was there growth of CRD in the absence of methicillin. Neither strain grew in medium SD without glucose, whether or not methicillin was present.

Washed suspensions of organisms of strains 8081 and CRD fermented glucose in the absence of methicillin at similar rates. From 5 µmol glucose, 10 µmol CO₂ (all due to acid production) was evolved in bicarbonate buffer. The presence of methicillin did not alter the rate or amount of gas evolution with either strain. No gas was evolved when glucose was fermented in phosphate buffer, though all the glucose disappeared and lactate was formed. Total lactic acid production was 12 µmol from 5 µmol glucose, and 2 µmol when no glucose was added to the suspended bacteria. With both strains, most of the lactate (about 75 %) was L-isomer (cf. Gordon & Doelle, 1975).

DISCUSSION

The first object of this study was to see whether a second methicillin-dependent substrain of *P. cerevisiae* 8081 could be isolated. Such a strain was obtained at the first attempt, and so the earlier isolation of a methicillin-dependent organism was not a unique unrepeatable event. However, the new dependent strain (MRD) was isolated less readily than strain CRD (White, 1968*a*); replica-plating was needed to make a final selection of MRD organisms. Strain MRD was distinct from CRD: acetate was not required for growth of MRD, and the rate of reversion of MRD to drug independence was very much higher. A possible interpretation of these findings is that strain MRD has become methicillin-dependent as a consequence of a single unstable mutation, whereas CRD has undergone either a different and more stable mutation, or else has suffered a second mutation that has stabilized the drug dependence. When strain CRD reverted to drug independence it maintained its methicillin resistance and also its nutritional requirement for acetate, so that CRD seems to differ from the parent strain by at least two (and probably more) mutational steps.

Resistance to methicillin increased stepwise during experiments with a mutagen (NTG). The first step gave resistance to 75 μg methicillin/ml, the second to 150 $\mu\text{g}/\text{ml}$; presumably at least one more step would be needed to give resistance to 400 $\mu\text{g}/\text{ml}$, as is found in MRD and CRD. This level of resistance to methicillin can be achieved without drug dependence being a necessary accompaniment (e.g. strain CR). Whether drug dependence might emerge without simultaneous gain of resistance is doubtful. Six attempts to isolate a methicillin-dependent organism that was not resistant were unsuccessful. Although methicillin-dependent organisms (of strain CRD) might have some selective advantage over methicillin-resistant but independent organisms (White, 1968*a*), this advantage may be so slight that dependent organisms might make up an appreciable part of a population only after a large number of transfers in the presence of the drug. Possibly methicillin-dependent organisms need a higher external concentration of the drug for growth than can be tolerated by a non-resistant organism. Strain CRD grew poorly with less than 40 μg methicillin/ml and about 100 $\mu\text{g}/\text{ml}$ was optimal. Other penicillins (e.g. benzyl penicillin, ampicillin) had only slight growth factor activity at concentrations that did not inhibit CRD, which was not resistant to these compounds (White, 1968*a*).

The lack of cross-resistance to benzyl penicillin is a striking feature of all the methicillin-resistant strains. The strain (8081 Pen^B) resistant to benzyl penicillin, however, had developed 15- to 30-fold resistance to methicillin as well as 6-fold resistance to benzyl penicillin. A possible interpretation of these findings is that resistance to methicillin might have developed by a mechanism (e.g. a specific diminished uptake) that did not confer resistance to other penicillins, while resistance to benzyl penicillin, which was more difficult to develop, arose by a different process that led to resistance against other derivatives of 6-amino-penicillanic acid.

No clear patterns emerged from the comparisons of the nutrition of *P. cerevisiae* 8081 and the methicillin-resistant substrains. The only common feature shown by all the resistant strains (whether methicillin-dependent or not) was a nutritional requirement for pantothenate, which was not needed by the parent strain. Pantothenate is a precursor of CoA, and this cofactor participates in the metabolism of acetate, which is disturbed in strain CRD (White, 1968*a*). Unlike the parent strain, CRD required acetate for growth yet was inhibited by concentrations of acetate higher than 1 % (w/v) unless methicillin were present. The need for acetate was not shown by strain MRD but was shown by methicillin-independent revertants of CRD, so that this property is not an inevitable consequence of methicillin

dependence. Whether acetate is toxic to strain MRD in the absence of methicillin has not been established.

The ability of strain 8081 to grow to moderate turbidity in the absence of lysine was unexpected, though mutants of other lactic acid bacteria have been found that do not require this amino acid (Vogel, Thompson & Shockman, 1970; Morishita *et al.* 1974). Strain CRD did not grow without lysine. This difference may be unrelated to drug dependence or it might reflect a greater requirement for lysine, which would be needed to synthesize the increased amount of peptidoglycan found in the wall of strain CRD (White, 1968*b*).

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