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Mutant of *Clostridium pasteurianum* That Does Not Fix Nitrogen

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A stable, non-nitrogen-fixing mutant of *Clostridium pasteurianum* W5 was isolated. The mutant has the same growth rate as the wild type on ammonia nitrogen, but is not able to grow on N_2. Cell-free extracts of the derepressed mutant are unable to fix nitrogen, or reduce the alternate substrates of nitrogenase such as cyanide, azide, or acetylene. Recombination with wild-type components from a diethylaminoethyl cellulose column indicates that the mutant contains component II, but is lacking component I activity.

At least two protein components derived from N_2-grown cells of *Clostridium pasteurianum* are necessary for nitrogen fixation in the assay system now in general use (9, 17). In addition, the requirement for a third component, which is present in N_2- or ammonia-grown cells, was recently indicated (15). Genetic analysis might resolve the question of the number of components required. If two mutants known to be lacking the same component could be shown to recombine to produce activity, the presence of a third protein might be indicated.

Moustafa and Mortenson (11) removed the activity of the cold-labile component from *C. pasteurianum* crude extracts, but as yet no independent assay has been developed for either individual component. The only available technique is the fractionation of the crude extract into the two components, followed by purification of each component using the other component for the N_2-fixation assay. Mutants with a lesion in the structural gene for one of the components may aid in the purification of nitrogenase, with the mutant crude extract being used to monitor the purification of the component which it lacks. Mutant analysis may also elucidate the mechanism by which N_2 and the alternate substrates of nitrogenase (e.g., cyanide, azide, and acetylene) are reduced.

Wyss and Wyss (20) and Green et al. (7) isolated mutants of *Azotobacter* unable to fix nitrogen. As cell-free enzyme preparations were not available at that time, further characterization of the mutants was not possible. Sorger and Trofimenkoff (14) reported isolation of nitrogenaseless mutants of *A. vinelandii*. Fisher and Brill (5) isolated and partially characterized mutants of *A. vinelandii*. It is believed that analysis of mutants for the nitrogenase of *Clostridium* as well as *Azotobacter* might provide useful information for elucidation of the mechanism of nitrogen fixation. This report describes the isolation and partial characterization of a mutant of *C. pasteurianum* unable to fix nitrogen.

MATERIALS AND METHODS

Chemicals. The diethylaminoethyl (DEAE) cellulose used was Whatman DE 52, (H. Reeve Angel, Inc., Clifton, N.J.). Penicillin G, lysozyme (EC 3.2.1.17), adenosine triphosphate (ATP), and ATP: creatine phosphotransferase (EC 2.7.3.2) were from Sigma Chemical Co., St. Louis, Mo.; penicillinase (EC 3.2.5.6) was from Calbiochem, Los Angeles, Calif.; and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was from Aldrich Chemical Co., Milwaukee, Wis. The gases used were acetylene, purified grade (The Matheson Co., East Rutherford, N.J.); helium, high purity grade (National Cylinder Gas Co., Chicago, Ill.); and nitrogen, prepurified (Airco, New York, N.Y.). Creatine phosphate was prepared in our laboratory by the method of Ennor (5).

Cultures and growth conditions. The prototrophic strain used throughout this work was *C. pasteurianum* W5, obtained as a spore stock in sterile soil from P. W. Wilson. Spores were heat-shocked for 10 min at 70 C in 10 ml of a medium containing 0.5% glucose and 0.5% yeast extract, and then transferred to modified Winogradsky's nitrogen-free medium (W; reference 18). This medium, supplemented with 400 μg of N per ml as $(NH_4)_2SO_4$ when desired (WN), was used throughout. Cultures were incubated anaerobically at 30 C, either on a rotary shaker under N_2 or sparged with N_2, unless otherwise indicated. Growth was followed turbidimetrically on a Klett-Summerson photoelectric colorimeter with a no. 64 filter. A standard curve of Klett units versus number of cells was used to determine the cell titer of a culture. Number of cells per milliliter was determined by a direct count in a Petroff-Hausser counting chamber. Plates contained 1.5% purified agar (Difco), W or WN, and 0.1% sodium thioglycolate, and were stored under N_2 until use. Cultures in the exponential

phase of growth (5×10^8 cells/ml) were diluted anaerobically in 0.85% saline, and dilutions were spread on the plates and incubated at 30 C in an atmosphere of 95% N_2, 5% CO_2 for 1 to 3 days.

Fractionation and assay of extracts. Cell-free extracts were prepared by the lysozyme method of cell disruption (19). Extracts were stored in liquid nitrogen until use, with no loss in activity during storage for up to 6 weeks.

The components of the assay system for reduction of N_2, N_3, C_2H_2, or CN^- were essentially the same as previously described (1, 4).

Fractionation of crude extracts was carried out on DEAE cellulose columns by methods described previously (2), except that 0.025 M tris(hydroxymethyl) aminomethane (Tris)-hydrochloride buffer, *p*H 7.4, was used as the equilibrating buffer and, after reduction of the column with dithionite, the dithionite was washed from the column with this buffer before addition of the extract. The columns were eluted sequentially with 0.025 M Tris buffer (*p*H 7.4), 0.15 M NaCl, 0.25 M NaCl, and 0.50 M NaCl. The NaCl solutions were prepared in 0.025 M Tris buffer (*p*H 7.4), and the 0.25 and 0.50 M NaCl solutions contained 0.1 to 0.2 mg of dithionite per ml. Component I was eluted with the 0.25 M NaCl solution, and component II with the 0.50 M NaCl solution.

Mutant isolation. A 100-ml culture of *C. pasteurianum* W5 was grown on WN to late exponential phase (10^9 cells/ml). *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was sterilized by filtration and added to the culture to a final concentration of 100 μg/ml, and incubation was continued for 30 min at 30 C on a rotary shaker. A 20-ml portion of the mutagenized culture was washed with 20 ml of 0.1 M phosphate buffer, *p*H 7.6. After suspending the cells in 20 ml of buffer, 4 ml was added to 100 ml of fresh medium and grown to early stationary phase. The culture was washed and transferred into fresh medium twice more; cells were then washed twice to remove any residual ammonia, transferred to W medium, and grown to 4×10^8 cells/ml (120 Klett units). Filter-sterilized penicillin G was added to a final concentration of 1,000 units/ml, and incubation was continued until the turbidity had dropped to 80 Klett units (about 4 hr). Filter-sterilized penicillinase (8,000 units/ml) and 400 μg of N per ml as $(NH_4)_2SO_4$ were added simultaneously at this point. The culture was recycled, with three intermediate transfers on WN, through three further penicillin selections.

After the fourth penicillin selection, the mutagenized culture was plated on limiting ammonia (W plus 60 μg of N per ml as $(NH_4)_2SO_4$). As soon as colonies were visible, the smallest colonies were picked onto WN plates by touching a fine platinum needle to the colony and stabbing into the ammonia plate to maintain a somewhat anaerobic atmosphere around the cells. Those which grew well were transferred to both W and WN plates in the same manner. Isolates which grew on WN but not on W plates were picked as presumptive mutants for nitrogenase and purified.

The presumptive mutants were grown on WN in flasks and then transferred to W. Growth proceeded for a short time until the residual ammonia from the inoculum was exhausted. Presumptive mutants which could not resume growth on N_2 after ammonia exhaustion were selected for further study as mutants unable to fix nitrogen.

RESULTS

By using the procedure described above, we isolated a mutant of *C. pasteurianum* W5 designated *C. pasteurianum* 1-8 that was unable to grow in the absence of ammonia. It was judged to be a mutant, rather than a contaminant picked up during manipulations, because (i) it has the same vegetative cell and spore morphology as the wild type; (ii) revertants able to fix N_2 appeared when cells of strain 1-8 were plated on nitrogen-free medium; and (iii) 1-8 has the same growth rate on full ammonia medium as does W5 (Table 1). The fact that the mutant grows at the same rate as the wild type on ammonia nitrogen but cannot use N_2 indicates that the genetic lesion is specific for nitrogen fixation.

Enzyme activity was examined in cell-free extracts of *C. pasteurianum* 1-8 to establish whether the mutation was involved with synthesis of nitrogenase. If this were not the case, the mutation might be concerned, for instance, with the permeation of nitrogen into the cell. Since ammonia is known to repress nitrogenase synthesis (10) and the mutant cannot grow on N_2, it would be derepressed under conditions of nitrogen starvation. A comparison of extract from starving cells of the mutant with extract from wild-type cells grown on N_2 might lead to misinterpretation. For this reason, a method was devised to derepress nitrogenase of W5 under conditions in which the cells were unable to divide. W5 extracts could then be compared to 1-8 extracts prepared under similar conditions.

A culture of W5 on W medium, containing a limiting ammonia concentration of 60 μg of N/ml, was inoculated from an ammonia-grown culture. The culture was sparged with helium, growth was followed turbidimetrically, and the presence of ammonia was detected by a Nessler spot test (16). Growth proceeded until ammonia was depleted but, because N_2 was not present, growth could not resume. Samples were taken at various times after ammonia exhaustion, and extracts were prepared and assayed for nitrogen fixation and acetylene reduction. Table 2 shows that nitrogenase is fully derepressed 6 hr after ammonia exhaustion, when compared with the activity of an extract from cells grown on N_2.

Cultures of 1-8 were grown in the same manner, and cell-free extracts were prepared 6 hr after ammonia exhaustion. Since the reductions of CN^- and N_3^- as well as C_2H_2 have been shown to be specific for nitrogen fixation (3, 8, 12, 13), assays for these reductions were included

TABLE 1. *Growth of mutant and wild type on nitrogen gas and ammonia*

	Nitrogen source	Doubling time (hr)
W5	NH^+_4	1.45
1-8	NH^+_4	1.44
W5	N_2	3.25
1-8	N_2	No growth

TABLE 2. *Nitrogen fixation and acetylene reduction during derepression of C. pasteurianum W5*

Nitrogen source for growth	Sparged gas	Hr after NH^+_4 exhaustion	Substrate:	N_2	C_2H_2
			Product[a]:	NH^+_4	C_2H_4
N_2	N_2			9.4	21.2
NH^+_4	He	0		1.1	0.5
NH^+_4	He	3		8.9	11.1
NH^+_4	He	6		12.1	19.5

[a] Activities are expressed as nanomoles of N_2 reduced (or C_2H_4 formed) per minute per milligram of protein.

TABLE 3. *Activities of extracts from derepressed C. pasteurianum W5 and 1-8 with several substrates*

Strain	Substrate:	N_2	CN^-	N^-_3	C_2H_2
	Product[a]:	NH^+_4	CH_4	NH^+_4	C_2H_4
W5 (N_2 grown)		18.8	2.2	33.2	21.2
W5 (NH^+_4 grown)		0.0	0.0	0.0	0.0
1-8		0.0	0.0	0.0	0.0

[a] Activities are expressed as nanomoles of product formed per minute per milligram of protein.

TABLE 4. *Assay for components I and II in crude extracts of the mutant with column fractions of the wild type*

Protein fraction[a]	C_2H_4 formed[b]
W5 (4.9)	75.0
W5I (0.23)	0.0
W5II (0.25)	3.6
W5I (0.23) plus W5II (0.25)	17.3
1-8 (2.9)	0.0
1-8 (2.9) plus W5I (0.23)	2.6
1-8 (2.9) plus W5II (0.25)	1.6

[a] Figures in parentheses indicate milligrams of protein per assay.

[b] Results expressed as nanomoles of product formed per minute. Acetylene reductions were incubated for 20 min at 30 C.

in the study. Extracts from 1-8 cells that were subject to the procedure that successfully derepressed W5 cells showed no activity for N_2 reduction nor for any of the alternate substrate reductions (Table 3). Assays of extract from W5 cells grown on ammonia are included as a negative control.

W5 crude extracts were fractionated on DEAE cellulose columns into components I and II of nitrogenase to test for recombination with 1-8. In this way it should be possible to demonstrate whether the mutant contains activity of one of the components. Acetylene reduction was the assay method of choice for these experiments because the greater sensitivity of this assay might show activities that N_2 reduction would not. Table 4 shows the results of one such experiment. W5 I had no activity when assayed by itself, whereas W5 II had a small residual activity. Recombination of W5 I and W5 II showed almost a fivefold increase over the activity of W5 II alone. *C. pasteurianum* 1-8 crude extract has no activity, but the recombination of 1-8 with W5 I is active, indicating that 1-8 contains component II. Strain 1-8 is apparently lacking component I, as crude extract of 1-8 inhibits the residual activity of W5 II (about 56% in this experiment), rather than showing recombination. In other similar experiments, this inhibition varied from 40 to 70%, depending on the amount of 1-8 extract used. It seems from these results that the mutant extract contains an inhibitor, perhaps hydrogenase (hydrogen: acceptor oxidoreductase, EC 1.12.1.1), which interferes with acetylene reduction (17). This complicates the interpretation that the mutant contains only component II activity, and may explain the low levels of 1-8–W5 I recombination, which is only about 15% of the W5 I–W5 II recombination.

To clarify these results, extracts of strain 1-8 were fractionated on a DEAE cellulose column in the same manner as W5, and the components were assayed in recombination with each other, and with components from W5 extract. It was hoped that, in this way, an inhibitor might be separated from components I and II, and levels of recombination might be increased. The results of this experiment (Table 5) are consistent with the crude extract recombinations. The activities of the wild-type column fractions are similar to those described above, whereas neither 1-8 I nor 1-8 II, nor the recombination of the two mutant fractions, has any activity. Strain 1-8 II shows recombination with W5 I, but the reciprocal recombination, 1-8 I plus W5 II, shows no increase over residual W5 II activity. In fact, W5 II activity is inhibited by 1-8 I as by 1-8 crude extract in this experiment, about 64%. If there is a specific inhibitor, it does not seem to be separated from component I of the mutant. Strain 1-8 II plus W5 I shows about 24% of W5 I plus W5 II recombination activity, which is a somewhat better re-

TABLE 5. *Fractionation of crude extracts from derepressed mutant and wild type*

Protein fraction[a]	C_2H_4 formed[b]
W5 (4.9)	63.5
W5I (0.69)	0.0
W5II (0.24)	3.3
W5I (0.69) plus W5II (0.24)	11.0
1-8 (4.3)	0.0
1-8I (0.58)	0.0
1-8II (0.32)	0.0
1-8I (0.58) plus 1-8II (0.32)	0.0
1-8I (0.58) plus W5II (0.24)	1.2
1-8II (0.32) plus W5I (0.69)	2.6

[a] Figures in parentheses indicate milligrams of protein per assay.

[b] Results expressed as nanomoles of product formed per minute. Acetylene reductions were incubated for 20 min at 30 C.

combination than in the crude extract experiment.

DISCUSSION

When W5 crude extract is chromatographed on DEAE cellulose columns, the fractions eluted by 0.25 M NaCl (component I) and 0.50 M NaCl (component II) can characteristically be seen as brown bands moving down the column. However, the 0.25 M NaCl fraction from columns of 1-8 extract is very light in color. The corresponding fraction from ammonia-grown cells also is light. This is perhaps an indication that component I is altered or not synthesized at all by strain 1-8, as the 0.50 M NaCl fraction closely resembles that from columns of W5 extract.

The possibility that an inhibitor is present in 1-8 extracts will be further investigated. By removing hydrogenase from 1-8 extracts, it should be possible to demonstrate whether hydrogenase in the mutant extract is responsible for the observed inhibition of W5 II by 1-8 crude extract. Vandecasteele and Burris (17) have shown that the hydrogenase in component I fractions from DEAE cellulose columns does inhibit nitrogen fixation and acetylene reduction in recombinations with component II, at high concentrations of component I. Since 1-8 seems to be lacking component I, the hydrogenase in 1-8 crude extract and the 0.25 M NaCl fraction from the columns may be sufficient to inhibit the residual activity of component II. Alternatively, 1-8 may synthesize a protein for component I which is inactive in nitrogen fixation, but which is capable of interacting with component II to inhibit the reaction.

The fact that 1-8 can produce component II in a nitrogen-free medium under helium is further evidence that this method of derepression is sufficient. However, there is N_2 present in the helium in quantities sufficient for induction, although not for growth, so that induction as a regulatory mechanism for synthesis of nitrogenase cannot be ruled out from these experiments.

Crude extract of derepressed cells of 1-8 may be useful in the purification of component I of nitrogenase, as the need for a pure component II of wild type is obviated, and component II is more stable in crude extracts than in column fractions (17). The DEAE column fraction which contains component II always has some component I contamination. With the use of mutants which do not contain component I, this contamination is not a problem, and complicated purifications of component II of the wild type would not be necessary to follow the further purification of component I.

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