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NITROGENASE

III. NITROGENASELESS MUTANTS OF *AZOTOBACTER VINELANDII*: ACTIVITIES, CROSS-REACTIONS AND EPR SPECTRA

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SUMMARY

Mutant strains of *Azotobacter vinelandii* that are unable to fix nitrogen were analyzed for their ability to reduce acetylene and oxidize dithionite. The activities of Components I (Fe–Mo-protein) and II (Fe-protein), the presence of antibody cross-reacting material to each of the components and the electron paramagnetic resonance (EPR) intensities at $g = 3.65$ also were examined in these strains. All mutant strains so far studied that are unable to reduce nitrogen, are also incapable of reducing acetylene or oxidizing dithionite. Representatives of various nitrogenaseless mutants have been characterized. Based on activity measurements they fall into three classes: those lacking both components (I^-II^-), those lacking Component I (I^-II^+) and those lacking Component II (I^+II^-). Many strains have extremely low levels of activity for either component, but in some of these strains, cross-reacting material is made for one or both of the components. The EPR at $g = 3.65$ correlates well with the activity for Component I in several of these mutant strains, but in four of the mutants there appears to be 10–20-fold higher amounts of paramagnetic center than the nitrogen-fixing activity in *in vitro* tests would indicate.

INTRODUCTION

Several workers have isolated mutants unable to fix nitrogen in *Azotobacter vinelandii*[1–4], *Clostridium pasteurianum*[5] and *Klebsiella pneumoniae*[6,7]. Gene transfer systems have been described for *Azotobacter vinelandii*[8] and *Klebsiella pneumoniae*[6,7]. Genetic studies were pursued in order to compliment the wealth of biochemical data on nitrogen fixation from various laboratories. In all organisms[9–15] so far examined, nitrogenase activity depends on the presence of both Component I (Fe–Mo-protein) and Component II (Fe-protein). When cells are grown in the presence of excess NH_4^+, no nitrogen reduction or acetylene reduction is observed[16].

This paper describes several techniques that have been applied in studies of mutant strains of *Azotobacter vinelandii* unable to fix nitrogen. The work is a preliminary investigation that has classified some mutants according to their Component I and II activities, cross-reactions to antibodies prepared against the components, activities with alternate substrates, electron paramagnetic resonance (EPR) at $g = 3.65$,

and complementation properties. These techniques can easily be applied in studies with other organisms capable of fixing nitrogen.

MATERIALS AND METHODS

Organisms

The wild-type organism on which these studies were performed is *Azotobacter vinelandii* OP (ref. 17). For ease of description and conformation to recommended nomenclature[18], *A. vinelandii* OP is referred to as strain UW, and mutant strains derived from strain UW are given numbers in the order in which they were isolated.

Chemicals

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine was obtained ftom Aldrich Chemical Co. ATP, creatine kinase (ATP:creatine phosphotransferase, EC 2.7.3.2), creatine phosphate, deoxyribonuclease I (deoxyribonucleate oligonucleotidohydrolase, EC 3.1.4.5), dithiothreitol and Tris–base were obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of analytical grade available commercially.

Media

A modified Burk's nitrogen-free medium[19] was used as the basal medium. When growth on NH_4^+ was required, 400 μg/ml of nitrogen as ammonium acetate was added. Unless otherwise specified, air is the gas phase and the incubation temperature is 30 °C.

Isolation of mutants

Cultures of strain UW were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine according to the method of Adelberg *et al.*[20], and mutants were isolated as previously described[3]. In some cases a penicillin enrichment was also used.

Growth conditions

Cultures were grown in 200 ml Burk's medium in 1-l baffle-flasks on a rotary shaker and the cell density was measured with a Klett–Summerson photoelectric colorimeter with a No. 64 filter. Limiting NH_4^+ (100 μg N_2/ml as ammonium acetate) was supplied and cells of mutant strains were harvested at 3 h after exhaustion of NH_4^+.

Assays

Preparation of extracts, purification of the components, and assays were described by Shah *et al.*[16]. Activities were calculated as indicated therein[16]. Protein concentrations were determined by the method of Bucher[21] or Lowry *et al.*[22]. Molybdenum determinations were performed by the method of Clark and Axley[23].

Antisera

Antiserum to Component II was prepared as previously described[24] for Component I. Component II used for preparation of antiserum was obtained by further purification of the DEAE-cellulose fraction by Sephadex G-200 column chromatography. Immunodiffusion experiments for screeming mutants for cross-reacting

material were conducted by a modification of the method of Ouchterlony[25]. The 50 mm × 12 mm petri dishes contain 1.5 % Bacto purified agar, 0.9 % NaCl, 0.02 % sodium azide in 0.025 M Tris–HCl buffer, pH 7.5. Wells of 3 mm diameter were placed 6 mm (center to center) from the center well to which antiserum is added. The lateral wells received crude extract (250–450 μg protein) or purified component sufficient to yield a precipitin line.

EPR spectroscopy

EPR spectroscopy with 100 KHz field-modulation was carried out at *x*-band essentially as previously described[24]. Preparation of samples for EPR spectroscopy was as described by Davis *et al.*[24].

RESULTS AND DISCUSSION

Extracts from all of the mutant strains that we have collected have been assayed for their ability to reduce nitrogen to ammonia with dithionite as the electron source. The mutant strains discussed in this paper are only those that have no discernible nitrogen-fixing ability *in vitro* as assayed by the standard technique for quantitating the amount of ammonia formed[19]. All of these mutants grow at the same rate as the wild type in liquid medium containing excess NH_4^+. The mutant phenotype presumably is caused by a single mutation because all mutant strains discussed in this paper are capable of spontaneously reverting to the wild-type, nitrogen-fixing phenotype.

Acetylene reduction is a very sensitive assay for nitrogenase activity[26] and consequently this assay was used for the more detailed analyses of the mutant strains. Another sensitive assay is the spectrophotometric ATP-dependent dithionite oxidation technique[27]. These activities require both protein components[9,10,14]. The sensitive acetylene-reduction assay indicates that mutant strains UW6, UW10 and UW38 still have a trace of activity (less than 0.9 % of the derepressed wild type, Table I, Column *a*). This basal activity normally cannot be detected by the standard assay for nitrogen conversion to ammonia due to the relatively low sensitivity of the assay. Extracts of the other strains do not show this trace activity even with higher concentrations of extracts in the assay mixture. With the dithionite-oxidation assay, the same results (only trace activity in strains UW6, UW10 and UW38) were obtained as with the assay by acetylene reduction.

Complementation with purified components for component activities in the mutants

All of the mutant strains in Table I, with the exception of strains UW91 and UW120 have very little Component I activity when the extracts from the mutant strains are titrated with Component II that has been purified from the wild type (Column *b*).

From Table I (Column *c*), it is apparent that strains UW6, UW10 and UW38 have activity for Component II as tested by titration with Component I that has been purified and crystallized from the wild type. Strains UW6 and UW10 have similar Component II activities, but strain UW38 has a specific activity nearly three times as high as these other two strains and this activity is greater than that found in strain UW that has been growing on nitrogen. The high activity of Component II

TABLE I

PROPERTIES OF MUTANT STRAINS

Specific activities expressed as nmoles ethylene formed/min per mg protein. For the EPR signal, the numbers are expressed as percent peak-to-peak amplitude of fully derepressed wild type; the random error is estimated to be about ± 0.3 (see text). n.d., not done.

Strain	*Number of representative mutant strains*	*Specific activities*			*Cross-reacting material*		*g = 3.65 EPR signal*
		Extract I		*II*	*I*	*II*	
		a	*b*	*c*	*d*	*e*	*f*
UW	—	60.2	74.5	68.1	+	+	100.0
UW1	8	0.0	0.8	0.0	−	−	0.7
UW3	8	0.0	3.5	0.0	+	−	4.0
UW6	4	0.1	0.1	51.5	−	+	2.7
UW10	8	0.6	0.6	60.0	+	+	6.9
UW38	1	0.2	0.2	165.7	−	+	5.4
UW91	1	0.0	83.0	0.0	+	+	96.0
UW112	1	0.0	0.1	0.0	+	+	4.0
UW118	10	0.0	0.9	0.0	+*	−	0.7
UW120	1	0.0	75.0	0.0	+	−	n.d.

* Extracts from strain UW118 and its representatives show a faint precipitin band.

in strain UW38 might be due to the lower ammonia pool resulting from nitrogen starvation, but the same high activity also would be expected in strains UW6 and UW10. One possibility for the lower activities of Component II in strain UW10 is that the altered Component I of the mutant still can compete with active purified Component I from the wild type. This would cause the observed activity to be lower than the actual activity of Component II in this type of mutant. Component II activity in strain UW6 cannot be explained in this manner because this strain contains no recognizable antibody cross-reacting material against Component I. However, Component I protein in strain UW6 might have been altered so that antigenicity is lost but still is able to compete with Component I from the wild type.

Origin of the residual Component I activity

All of the strains in Table I have at least a trace of Component I activity which normally can be detected only by the acetylene-reduction or dithionite-oxidation assays. It is not possible that Component II in a crude extract is capable of reducing acetylene at a very low rate because purified Component II has no activity by itself[28]. To test whether nitrogen reduction also can occur in these mutants at this low rate, the time of incubation for the assay was 5 h instead of 15 min. With this technique, a small amount of nitrogen-fixing ability was observed in strain UW38 (0.05 nmole N_2 reduced/min per mg protein) that correlates with the low acetylene-reduction results. The trace activity in strain UW38 can therefore be detected by nitrogen fixation as well as acetylene reduction.

A test to determine if this trace activity is independent of Component I was to add antiserum that had been prepared against pure, crystalline Component I to an extract of strains UW6 or UW38. This cross-reacting material completely abolishes the residual activity in extracts of these strains even in the presence of added Component II that had been prepared from the wild type. It seems, therefore, that Component I is responsible for the low activity.

Another test used to demonstrate that the trace of activity actually is due to a small amount of Component I, was to fractionate an extract of strain UW38 on a DEAE-cellulose column by using the same techniques as those used to fractionate the components from the wild type[9]. The results in Table II clearly demonstrate that Component II from strain UW38 is not active by itself and addition of Component I fraction from strain UW38 generates about 1 % activity compared to the addition of Component I of wild type. The basal activity is not due to reversion by spontaneous mutation because fewer than 20 colonies are seen after 7 days when $1 \cdot 10^8$ cells from cultures of these mutant strains are plated on nitrogen-free plates.

TABLE II

ACTIVITIES OF COMPONENTS I AND II FROM DEREPRESSED MUTANT STRAIN UW38

Figures in parentheses indicate mg protein fraction per assay. Activities are expressed as nmole of ethylene formed/min per mg protein.

Protein fraction	*Ethylene formed*
I (0.25) from UW38	0.00
II (0.16) from UW38	0.00
II (0.16) from UW38 *plus* I (0.25) from UW38	2.66
II from UW38 (0.032) *plus* I (0.06) from UW	237.00
I from UW (0.6)	0.00

Complementation of extracts from the mutants

All of the mutant strains that have lost Component I activity, but still have retained an active Component II ($I^- II^+$) are capable of yielding high levels of acetylene- and nitrogen-reducing activities when an extract from such a strain is mixed together with an extract from strains UW91 or UW120 ($I^+ II^-$). Table III contains the results of acetylene-reducing activity in cell-free extracts of strains UW10 and UW91. When the two extracts are mixed, high activity is observed. No other complementation was noticed with many combinations of extracts from two or more mutants. Extracts from representatives from each of the mutant classes were fractionated on DEAE-cellulose columns to separate the two components. When these fractions are mixed in various combinations, only Component I from strains UW91 and UW120, and Component II from the mutant strains UW6, UW10 and UW38 complement to yield activity.

TABLE III

COMPLEMENTATION OF EXTRACTS FROM DEREPRESSED MUTANTS

Numbers in parentheses are mg protein per assay. Activity expressed as nmoles ethylene formed/min.

Source of extract	*Activity*
UW91 (3.0)	0.0
UW10 (3.5)	1.3
UW91 (3.0) *plus* UW10 (3.5)	212.4

Antibody cross-reacting material against the components

Cross-reacting material for Component I, when placed in the center well of an Ouchterlony plate gives a precipitin band when pure Component I is in the outer well (Fig. 1). An extract from nitrogen-grown cells of strain UW also forms this band, whereas an extract from strain UW that has been grown in the presence of excess NH_4^+ does not form the precipitin band. It is known[16,19] that nitrogenase is repressed when excess ammonia is present. Purified Component II does not form a precipitin band. Extracts from mutant strains (Fig. 1, Table I) such as strains UW3, UW10 and UW91 placed in the outer well are able to form a discernible precipitin band. This is an indication that the inactive protein (or active protein in the case of strain UW91) still can be recognized by the antibodies. Extracts from mutant strains UW1, UW6 and UW38 are not capable of forming visible precipitin bands (Fig. 1, Table I, Column *d*). In these strains, Component I either is completely missing or is damaged sufficiently so that the resulting structure can no longer be recognized by the antibodies. Extracts from strains UW112 and UW118 also form precipitin bands against Component I antibodies (Table I, Column *d*). The precipitin reaction allows us to detect the component proteins when present at greater than 1 % of the nitrogen-grown wild-type levels. The 1 % level shows a faint response; about equivalent to the response found in extracts of strain UW118.

In Fig. 2, the center well of the Ouchterlony plate contains antiserum against purified Component II. The purified Component II is only about 98 % pure and the antibodies made against this preparation also form a light precipitin band against extracts from ammonium-grown cells. This band does not interfere with the analyses because it clearly is distinct from the Component II band which shows a stronger precipitin reaction and is closer to the center well. Again, extract of wild-type cells that have been grown on nitrogen and purified Component II cause the heavier precipitin band to be formed. Extracts from repressed wild-type and derepressed mutant strains UW1 and UW3 show no Component II-specific precipitin band. All of the mutant strains that have activity for Component II (UW6, UW10 and UW38) also

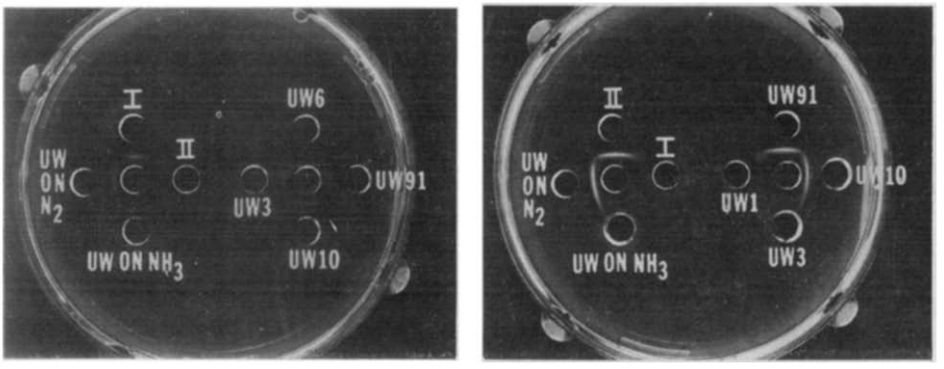

Fig. 1. Ouchterlony plate for cross-reaction with serum prepared against Component I. The center wells contain anti-Component I serum (32 μg protein). The outer wells contain extracts (250 μg protein) from derepressed cells or purified Component I (5 μg protein) or purified Component II (50 μg protein).

Fig. 2. Ouchterlony plate for cross-reaction with serum prepared against Component II. The center wells contain anti-Component II serum (32 μg protein). The protein concentrations in the outer wells are described in the legend for Fig. 1.

are capable of forming a precipitin band (Table I, Column *e*). Even though Component II of strain UW91 is inactive, it still retains the ability to cross-react. No cross-reaction is seen for Component II of strain UW120. The inactive Component II in strain UW91, therefore, still is present, but is either absent or unrecognizable in strain UW120. Extract from strain UW112 has antibody-recognizable Component II, whereas strain UW118 does not produce a cross-reacting protein (Table I, Column *e*).

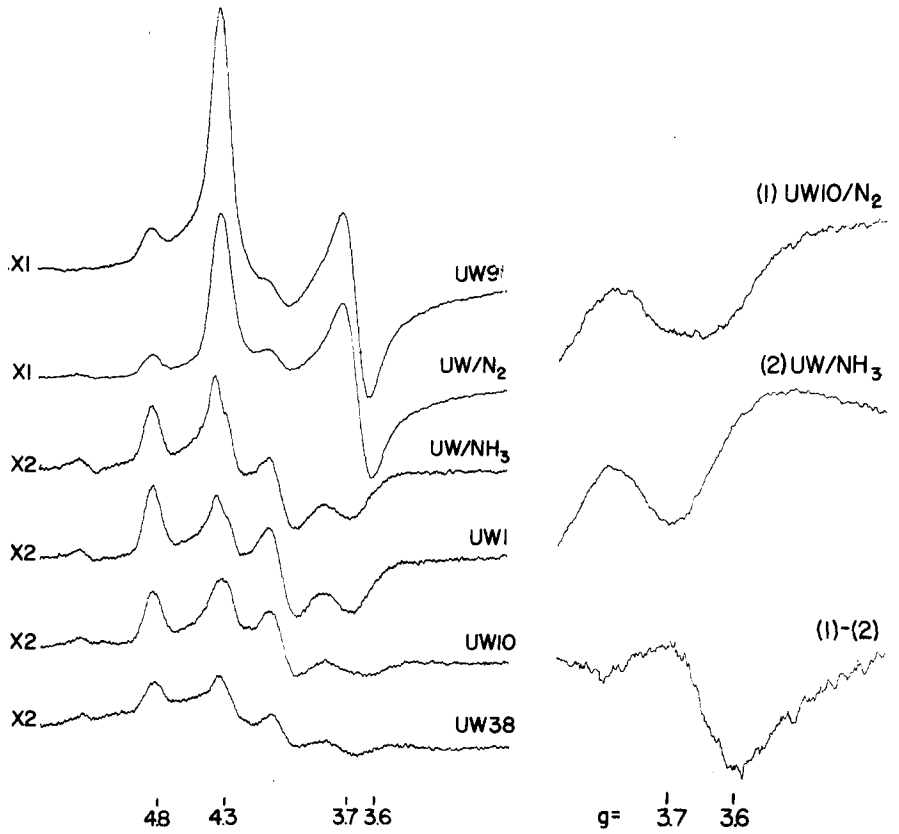

Fig. 3. EPR spectra of whole cells of UW and representative mutants, in the $g = 3$ to $g = 5$ region. Top to bottom: Mutant UW91, derepressed; UW, derepressed; UW, repressed; UW1, UW10, UW38, all derepressed, as described in the text. Note particularly the differences in the $g = 3.6$ to $g = 3.7$ portions of the spectra; *c.f.* also Fig. 4. The ordinate is an arbitrary linear function of the first derivative of the microwave absorption; the abscissa is linear in G. The positions of several values of g are indicated along the abscissa. Conditions of EPR spectroscopy: microwave power, 9 mW; microwave frequency, 9.17 GHz; modulation amplitude, 12 G, modulation frequency 100 kHz; field scan rate, 400 G/min; time constant, 0.25 s; temperature, 13 °K. The relative final amplifier gains are indicated along the left edge of the figure.

Fig. 4. Computer subtraction of signals from repressed UW cells (middle) from those of derepressed UW10 (top) to give difference spectrum in the $g = 3.6$ to $g = 3.7$ region (bottom). Conditions of EPR spectroscopy were the same as in Fig. 3 except that the field scan rate was 100 G/min and the amplifier gain and dispersion of the spectra were increased to give a magnified presentation. A single spectrum from each sample was put into the memory of a Varian C-1024 computer of average transients, with opposite signs of the ordinate so that the resultant spectrum stored in the computer was the difference between the two original spectra. The difference spectrum is displayed at approximately 2-fold higher gain than the original spectra.

EPR spectra of whole cells from derepressed mutant strains

In Fig. 3 are shown the EPR spectra obtained with packed whole cells of strain UW, repressed and derepressed, as well as the spectra of derepressed cells of UW1, UW10, UW38 and UW91. These spectra cover the region $g = 3$ to $g = 5$ where the resonances due to Compoment I are seen[24]. Near $g = 4.3$, resonances, presumably due to Fe^{3+} in a rhombic environment, interfere severely with the detection of small amounts of component I. On the other hand, near $g = 3.65$ the presence of approx. 5 % Component I in UW10 and UW38 (*cf.* Table I) results in a flattening of the signal that is present, for example (Fig. 3), in the repressed UW cells and in strain UW1. We did a computer analysis of the spectra in this region, subtracting the repressed UW spectrum from the derepressed UW10 spectrum. The results are shown in Fig. 4. The residual signal resembles that of derepressed UW in this region, with turning points at $g = 3.6$ and $g = 3.7$. This finding led us to the following procedure for estimating the amount of Component I in the mutants: We recorded the mutant spectra in this region at high gain, and calculated the fractional amount of the signal from derepressed UW that would have to be added to the signal from repressed UW recorded under the same conditions, to yield the observed spectra from the mutant. These fractional amounts are given as percentages in Table I. In addition to the estimated random errors of $\pm$ 0.3 % in this procedure, systematic errors could arise if signals other than those of Component I varied during growth or sample preparation. However, inspection of Table I shows that for mutants UW1, UW3, UW91 and UW118, the agreement between activity and the EPR signal is fairly good. We are convinced from studies of purified Component I as well as from repression and derepression studies on UW (ref. 24), that the signal at $g = 3.65$ (as well as signals at $g = 4.3$ and 2.01, obscured by other signals in whole cells) represents a transition metal complex in active Component I.

The fact that UW6, UW10, UW38 and UW112 show signs of having 10–20-fold more EPR center than detectable activity (see Table I) may have some importance. We foresee the following possibilities for this behavior: (1) The EPR estimation may be wrong due to the presence of extraneous signals in some of these mutants. (2) The activity measurements may be too low, if in analogy to the mutant proteins of certain temperature-sensitive mutants[29], the Component I of these mutants is unstable once it is freed from the cellular milieu. (3) The mutation may be in the site where interaction of Components I and II takes place. Clearly a different combination of the above considerations may explain each mutant of this category, but we do not think any of the explanations by themselves overwhelmingly plausible. We are at present attempting to resolve these questions by purification of large amounts of the mutant Components I.

Sites of possible genetic lesions that might be responsible for the phenotypes of the mutant strains

Because molybdenum is found in Component I, we thought that it might be possible that a strain deficient in its ability to assimilate molybdenum might have the phenotype of inactive Component I. To test this possibility we washed and centrifuged whole cells of derepressed mutant strains with 0.025 M Tris–HCl buffer, pH 7.4, at room temperature. Extracts were made by the usual osmotic shock method[16]. The wild type as well as representatives from each of the mutant classes were found

to contain 0.2–0.4 mg molybdenum per g protein (strains UW112, UW118 and UW120 were not tested). Therefore, for these mutant strains the defect responsible for their failure to grow on nitrogen is not a generalized defect in molybdenum uptake.

Another type of mutation generating the phenotype of good growth on ammonia but no growth on nitrogen could be a lesion in a gene involved in ammonia assimilation such as those reported in *Klebsiella pneumoniae*[30]. However, these *K. pneumoniae* mutants still have high activities for acetylene reduction (Nagatani, H. and Wilson, P.W., unpublished). Therefore it is not probable that any of the mutants discussed in this paper actually are of the type in which ammonia assimilation is disrupted. Because dithionite is the electron source for our assays, the natural electron-donating system is bypassed. A mutant strain that is defective in a nitrogenase-specific electron-transfer protein should be unable to utilize nitrogen, but should still be capable of fixing nitrogen *in vitro*. We have not yet found such a strain.

One striking result is that all of these mutant strains have a trace of Component I activity. This might merely be the consequence of the nature of the missense mutations caused by *N*-methyl-*N'*-nitro-*N*-nitroguanidine. However, one would expect some of these mutations to occur in a locus that is critical to the function of Component I (for example, an active site). Another possibility is that this residual activity somehow is necessary for growth on NH_4^+; in this case, mutants that completely lack Component I will not be selected by our techniques. This low activity also might be the result of a duplication of genes that specify Component I. Sadoff *et al.*[31] have reported that *A. vinelandii* contains ten times as much DNA per nucleoid as does *Escherichia coli* and that it has been difficult to obtain auxotrophs of *A. vinelandii*.

Mutant strains that have essentially no activity for either component and also have no cross-reacting material for either component (strain UW1) might be the result of mutation in a regulatory gene. Strains UW6, UW10 and similar mutants probably are caused by a mutation in a gene that codes for Component I. Likewise, strains UW91 and UW120 have a mutation in a gene that specifies Component II. It is more difficult to rationalize the origin of the phenotypes of mutant strains UW3, UW112 and UW118. These might be the results of polar mutations.

Transduction, transformation[8] and studies of revertants of these mutant strains should help in the actual understanding of the genotypes of these and other strains. The techniques described in this paper are also being applied to work with *K. pneumoniae*

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