Vol. 89, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 27, 1979 Pages 659-663

EFFECT OF GROWTH CONDITIONS ON THE SYNTHESIS OF NITRATE REDUCTASE COMPONENTS IN CHLORATE RESISTANT MUTANTS OF ESCHERICHIA COLI K 12

P. FORGET

Laboratoire de Chimie Bactérienne, C.N.R.S., 31, chemin Joseph Aiguier, 13274 Marseille Cedex 2 (FRANCE)

Received April 21,1979

SUMMARY. The chlorate resistant mutants of Escherichia coli synthetize, in variable quantities, proteins which give immunocomplex with specific nitrate reductase antiserum. The biosynthesis of these cross reacting materials presents the same type of regulation as nitrate reductase of the wild type. C.R.M. biosynthesis is repressed by oxygen and even in the presence of nitrate, the oxygen inhibition is not reversed with chlorate mutants and wild type. With anaerobically grown cells, nitrate acts as an inducer and increases the amount of antibody-precipitable material, three times in mutants and even four times with Chl-E .

INTRODUCTION

In Escherichia coli,the two electron reduction of nitrate to nitrite is catalyzed by the ironsulfurmolybdoprotein nitrate reductase, an enzyme of high molecular weight (320,000 daltons) (1) localized in the cytoplasmic membrane. Previous studies have demonstrated that the enzyme is induced by anaerobic growth in the presence of nitrate and repressed by oxygen (2) (3) (4).

In the presence of chlorate, the anaerobic growth of denitrifying bacteria is inhibited and only mutants, deficient in respiratory nitrate reductase activity exhibit growth. Thus mutants can be selected on the basis of resistance to chlorate (5). In Escherichia coli, the mapping of the genes has revealed different types of chlorate resistant mutants (6,7,8,9) which present the same phenotype : a simultaneous loss of nitrate reductase and formate dehydrogenase activities for chl-A, chl-B, chl-E and chl-D mutants. Different from the other mutants, chl-C, deficient in nitrate reductase activity, presents a formate dehydrogenase activity which is not affected by the mutation. chl-C is the unique specific mutant for nitrate reductase isolated at this day (8,10,11).

These mutations have been interpreted earlier as a result of an alteration in the cytoplasmic membrane (10,12). However since nitrate reductase and formate dehydrogenase are two molybdoproteins (1, 13) and since chl-D mutants form normal levels of nitrate reductase activity when they are supplemented with Mo (14), the hypothesis of a Mo involvement is now proposed (15, 16).

Using specific anti-nitrate reductase immunoserum, MacGregor (17) found that chlorate mutants contained a cross reacting material (CRM) which is located

0006-291X/79/140659-05$01.00/0

in the cytoplasmic membrane. For our part, using specific antiserum, we intend to analyze the different chlorate resistant mutants to determine whether the biosynthesis of CRM is repressed by oxygen and induced by nitrate as occurs with the nitrate reductase in the wild type.

MATERIALS AND METHODS

Growth conditions and crude extract preparation.

Escherichia coli strain K 12 PA 601 and chlorate mutants were grown in a rich medium at 37°, the growth conditions were the same for all the organisms and have been described (9). The chl-A, chl-B, chl-C and chl-E mutants were isolated and mapped by Puig et all (6) and Casse (7) ; they do not reduce nitrate to nitrite and only chl-C produces gas from formate. Cells were broken in a French pressure cell in 50 mM sodium phosphate buffer pH 7 and the crude extracts were prepared by centrifugation at 30,000 g for 20 min.

Assays.

Nitrate reductase was tested as previously reported (1) by a manometric method using benzyl viologen as electron carrier, activity is expressed as µmole NO_3^- reduced/h/mg of protein. Protein concentration was estimated by the Folin phenol method of Sutherland et al. (16) with bovine serum albumin as protein standard.

Preparation of antiserum.

Purified nitrate reductase (1) was used as the antigen. Homogeneity of the antigen was confirmed by polyacrylamide gel electrophoresis (19). Immunization of rabbits was performed by three sequential injections of antigen (1 mg) incorporated into complete Freund's adjuvant. The time between each injection was one week and 10 days after the last, a fourth injection without adjuvant was made with only 0.50 mg of protein. Ten days later the animals were bled by heart puncture, the collected serum was filtered on Millipore filters (0,45 µm pore size) and stored at -20°. The specificity of antisera was tested by double diffusion in agar gel. As the antigen, we used two different nitrate reductase solutions, one partially purified and the second homogeneous as demonstrated by gel electrophoresis (19). In all cases, we observed a single band of immunoprecipitate with a complete fusion line Other proteins contained in the partially purified solution of nitrate reductase did not produce any visible precipitin reactions.

Estimation of cross reacting materials (C.R.M.).

Estimation of C.R.M. was determined by precipitin reactions performed in two successive steps. First, mutant crude extracts were incubated with antiserum, followed by centrifugation to remove the antigen antibody complex. In the second step, excess antiserum was estimated by a precipitin reaction in the presence of soluble purified nitrate reductase.

The quantities of purified nitrate reductase and antiserum were adjusted to correspond to their equivalence points. Under these conditions, the excess of soluble nitrate reductase, found after the second precipitin reaction, corresponded to the quantity of C.R.M. present in the mutant crude extract. The quantity of mutant crude extract which precipitates all the antiserum is used as reference to compare the content in C.R.M. of mutants.

Incubation time was one hour at room temperature. Precipitations were performed in 1 ml of a solution containing : 100 µmoles sodium phosphate

buffer pH 7 ; 152 µmoles NaCl and equal quantities of antiserum and nitrate reductase. In each assay, the quantity of nitrate reductase corresponded to 0.5 µmoles NO_3^- reduced per minute and the quantity of antiserum corresponded to the equivalence point. The precipitate, formed during the antigen antibody reaction, was still enzymatically active and reduced nitrate to nitrite. Consequently the precipitate was discarded by centrifugation at 30,000 g for 20 min. before the excess of nitrate reductase was determined. In presence of an excess of antiserum, the supernatants assayed for nitrate reductase showed less than 3 % of the initial enzyme activity.

RESULTS AND DISCUSSION

Specific antiserum to purified nitrate reductase contains precipitin antibodies which can be used to estimate the amount of nitrate reductase. We have verified on the wild type that the immunological estimation agrees with the estimation based on the enzyme activity and that the specific activity and the yield of antigen evolved in the same proportions.

To study the influence of growth conditions on the biosynthesis of C.R.M., we have used four chlorate resistant mutants (6,7). In anaerobiosis and in presence of nitrate all these mutants synthetize C.R.M. localized in the cytoplasmic membrane. A fifth mutant, chl-D, has been discarded because of a too high residual nitrate reductase activity which makes insecure the estimation of C.R.M. by our method.

When aerobically grown cells are used, we observed the influence of oxygen on the biosynthesis of C.R.M. No antibody precipitable material is detectable in the crude extracts and even if nitrate is added to the growth medium, wild strain and chlorate mutants do not give precipitin reactions (Table I). The inhibition by oxygen of the nitrate reductase biosynthesis as well as C.R.M. is not reversed by nitrate. We have verified when no precipitation occurs that no soluble immunocomplex is formed and that all the antiserum used for the estimation is still present and can react against a new added antigen.

The oxygen inhibitor effect on the biosynthesis of nitrate reductase has been demonstrated previously (2,3,4). With the help of a different technique, we confirm the oxygen inhibition. These results also indicate that the presence of oxygen inhibits not only the synthesis of nitrate reductase but also the synthesis of a potential precursor which might occur in an inactive form.

If the presence of nitrate is without effect in aerobiosis, this compound acts as an inducer in the absence of oxygen. Anaerobically grown cells have a higher amount of nitrate reductase and C.R.M. when nitrate is added to the growth medium. The induction is visible in both cases with wild strain and chlorate mutants, six times in the first case and only three times in the second. With chl-C mutant the results are different. The precipitation reactions occur only with anaerobically grown cells in the presence of nitrate and even in this conditions the amount in antibody-precipitable material is less important (Table I). With this mutant using

TABLE I. Precipitation reactions with crude extract prepared from cells grown in different conditions.

Growth conditions	STRAINS					N.reductase activity of W.T. (1)
	Wild type	chl-A	chl-B	chl-C	chl-E	
Anaerobiosis plus NO_3^-	1	3	5	16	4	300
Anaerobiosis minus NO_3^-	6,6	10	16	ND	16	50
Aerobiosis plus NO_3 and minus NO_3	ND	ND	ND	ND	ND	ND

Each system contains the same quantity of antiserum.
The numbers give the weight of crude extract protein in mg used to reach the equivalence point.

ND. Not detectable

(1) Nitrate reductase activity determined by a manometric method (1) ; activity is expressed as μmoles NO_3^- reduced/h/mg of protein.

other growth conditions, precipitation was not observed clearly. It is possible that in absence of inducer there is not enough C.R.M. to be detected by our method. With the other mutants, the presence of nitrate increased the content in C.R.M. The equivalence point is reached with three times less protein of cells grown anaerobically in the presence of nitrate than in its absence. With the wild strain the induction by nitrate gives seven times more nitrate reductase. If the mutation corresponds to an alteration of a polypeptide chain, it is possible that the mutation affects also the antigenicity of the precipitable protein and modify the immunological response. An other reason to explain these differences, is that in mutant strains nitrate reductase is less accessible to the antibody. Further experiments now in progress on the purification of chl-A nitrate reductase do not indicate that the presence of a detergent increases the amount of antigen in this mutant.

A previous report (17) mentioned the presence of C.R.M. localized in the cytoplasmic membrane of chlorate resistant mutants. Compared to the wild type, these results indicate that chl-C and chl-E contain a lower amount of C.R.M. and chl-A and chl-B a normal amount. For our part, we obtained a lower amount with the four mutants tested. In the above mentioned report, it is pointed out that mutants make defective enzymes which are easily cleaved by proteolysis and immune precipitations could be influenced by such a fragmentation. Nevertheless the lower amount of C.R.M. which has been observed in different chlorate mutants has not been still explained.

If quantitatively the immune responses are different, qualitatively we observe that chlorate resistant mutants synthetize proteins which share with nitrate reductase, antigenic determinants and the same type of regulation for their biosynthesis.

REFERENCES

1. Forget, P. (1974) Eur. J. Biochem., 42, 325-331.
2. Stickland, H.T. (1931) Biochem.J., 25, 1543.
3. Collins, F.M. (1955) Nature, 175, 173-174.
4. Pichinoty, F. and d'Ornano, L; (1961) Nature, 191, 879-881.
5. Piéchaud, M., Puig,J., Pichinoty, F., Azoulay, E. et Le Minor, L. (1967). Ann. Inst. Pasteur, 112, 24-37.
6. Puig, J., Azoulay, E. (1967) C.R.Acad.Sci.Paris, 264, 1916-1918.
7. Casse, F. (1969) Biochem. Biophys. Res. Commun., 39, 429-436.
8. Guest, R.Z. (1969) Mol. Gen. Genet., 105, 285-287.
9. Taylor, A.L., et Trotter, C.D. (1972) Bacteriol. Rev., 36, 5&4-524.
10. Azoulay, E., Puig, J. and Pichinoty, F. (1967) Biochem. Biophys. Res. Commun., 27, 270-274.
11. Pichinoty, F., Chippaux, M. (1969) Ann. Inst. Pasteur, 117, 145-178.
12. MacGregor, C.H. and Schnaitman, C.A. (1971) J. of Bacter., 108, 564-570.
13. Enoch,H.G. and Lester, R.L. (1975) J.B.C., 250, 6693-6705.
14. Sperl, G.T. and DeMoss, J.A. (1975) J. Bact., 122, 1230-1238.
15. MacGregor, C.H., Schnaitman, C.A. (1975) J. Bacteriol., 112, 388-392.
16. Dubourdieu, M. (1976) B.B.R.C., 70, 766-773.
17. MacGregor, C.H. (1975) J. of Bacteriology, 121, 1117-1121.
18. Sutherland, E.W., Cori, C.E., Haynes , R. and Olsen, N.S. (1949) J. Biol. Chem., 180, 825-830.
19. Davis, B.J. (1964) Ann. N.Y.Acad.Sci., 121, 404-410.