JOURNAL OF BACTERIOLOGY, July 1989, p. 4092–4094
0021-9193/89/074092-03$02.00/0

Vol. 171, No. 7

Temperature Inhibition of Siderophore Production in *Azospirillum brasilense*

ANAND K. BACHHAWAT†* AND SUDHAMOY GHOSH

Department of Biochemistry, Bose Institute, Acharya, J. C. Bose Centenary Building, P-1/12 C.I.T. Scheme VII M, Calcutta 700 054, India

Received 18 November 1988/Accepted 7 April 1989

The effect of growth at 42°C on the different components of the siderophore-mediated iron transport that are induced by iron limitation in *Azospirillum brasilense* was examined. Biosynthesis of the siderophore spirilobactin was strongly inhibited (20-fold) by growth at 42°C, whereas the transport of iron by the ferric-spirilobactin transport system and the induction of the iron-regulated outer membrane proteins were unaffected.

Azospirillum brasilense is a gram-negative, nitrogen-fixing soil bacterium that symbiotically associates with the roots of several cereal grasses and enhances their growth (6, 12). Although this microorganism has been identified mostly in tropical soils, it is unable to fix nitrogen at temperatures above 40°C (5). We have recently shown that, under conditions of iron limitation, this bacterium initiates biosynthesis of its siderophore, spirilobactin, and induces several new high-molecular-weight proteins in the outer membrane and a high-affinity transport system for iron mediated by spirilobactin (2, 3). Siderophores have been identified in a large number of microorganisms, and in a few cases, siderophore biosynthesis has been shown to be inhibited by elevated temperatures (4, 8–10, 13). As siderophore production is important to meet increased iron requirements (7), we have sought to explore a possible link between temperature inhibition of nitrogen fixation and iron assimilation. With this end in view, we have examined how the different components of the high-affinity iron transport system mediated by spirilobactin are affected by elevated temperatures in *A. brasilense*. The results of this study are described in this report.

A. brasilense RG used for these experiments was described earlier (11), and the bacterium was grown on fructose minimal medium (FMM) (3) without added iron (low iron) or with 36 μm $FeCl_3$ (high iron). In some experiments, the bacterium was grown in Luria broth-succinate medium (2). Spirilobactin was assayed by the method of Arnow (1, 3).

Effect of temperature on growth and spirilobactin production. The growth of *A. brasilense* RG in low-iron medium and the siderophore production of these cultures at 32 and 42°C are shown in Fig. 1. Practically no spirilobactin was observed in the cultures grown at 42°C. The possibility that spirilobactin itself was unstable at 42°C can be ruled out by the fact that the siderophore was found to be quite heat stable (at 45°C with a rotary evaporator) in our earlier studies (3). Although initial growth was better at 42°C, a drastic inhibition in growth occurred at the time when cultures at 32°C were beginning to produce spirilobactin, which seems to suggest that the growth inhibition at 42°C was due to the inability to produce spirilobactin and a consequent nonavailability of iron. To examine whether such a supposition was indeed valid, we examined the growth of *A. brasilense* at these two temperatures in high-iron medium. A much better growth of the cultures at 42°C was observed in the high-iron medium, compared with that observed in low-iron medium (Fig. 2). Such striking differences in growth between the high- and low-iron media were not observed in cultures grown at 32°C. This indicates that the availability of iron becomes a very critical requirement at 42°C. However, in the presence of high iron, the final growth of the cultures at 42°C was less than that of cultures grown at 32°C, although

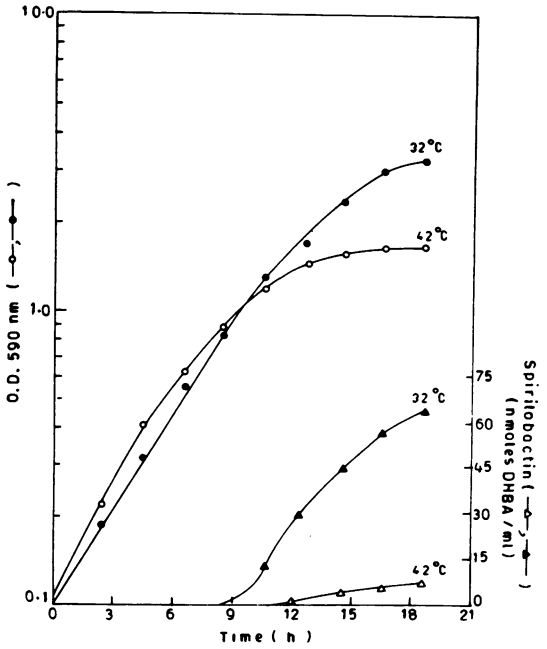

FIG. 1. Effect of temperature on growth and spirilobactin production of *A. brasilense* in low-iron medium. *A. brasilense* RG cells were grown overnight in FMM without iron, washed, and inoculated into the same medium (i.e., without $FeCl_3$) to a cell density of 0.1 OD_{590} units. The cells were incubated with shaking at 32°C and 42°C. Spirilobactin production of these cultures at 32°C and 42°C was measured as described in the text.

* Corresponding author.
† Present address: Department of Microbiology & Sanitation, Central Food Technological Research Institute, Mysore 570 013, India.

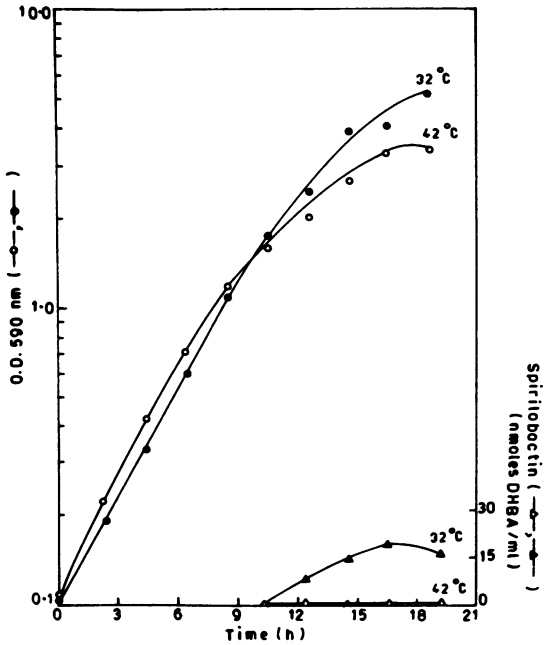

FIG. 2. Effect of temperature on growth and spirilobactin production of *A. brasilense* in high-iron medium. *A. brasilense* RG cells were grown overnight in FMM without iron, washed, and reinoculated into FMM containing 36 μM $FeCl_3$ to a cell density of 0.1 OD_{590} units. The cells were incubated with shaking at 32°C and 42°C. Spirilobactin production of these cultures at 32 and 42°C was measured as described in the text.

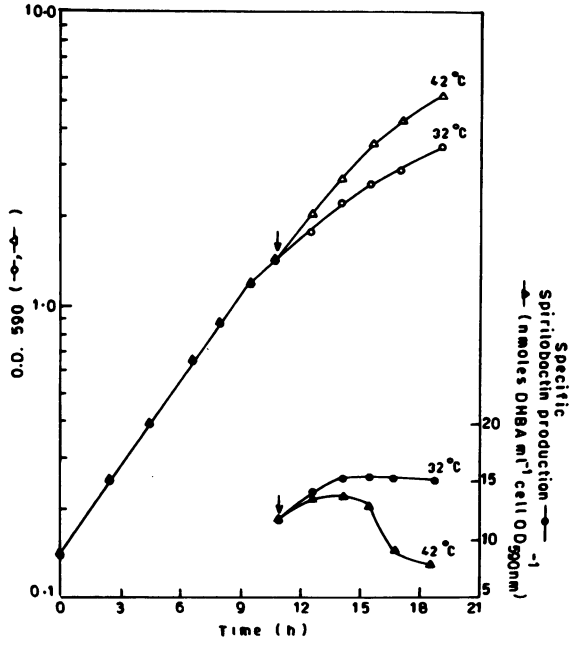

FIG. 3. Effect of temperature shift on the growth and spirilobactin production of *A. brasilense*. *A. brasilense* RG cells were grown overnight in FMM without iron, washed, and reinoculated into the same medium (i.e., without $FeCl_3$) to a cell density of 0.1 OD_{590} units. The cells were incubated with shaking at 32°C and, when siderophore production commenced, one batch was shifted to 42°C at the point indicated (↓) when it was allowed to grow also with shaking. Spirilobactin was estimated as described in the text.

the initial growth was better. While examining the supernatants in these high-iron cultures for spirilobactin, we observed that even in the presence of high iron, the cultures grown at 32°C produced a basal level of spirilobactin (15 nmol/ml). This is possible because of precipitation of iron after prolonged shaking in an aerobic atmosphere. Since cultures grown at 42°C were unable to produce even this basal level of spirilobactin, their growth rate diminished at the point where spirilobactin became necessary.

Effect of temperature shift on spirilobactin production. To investigate the nature of this inhibition of spirilobactin production at higher temperatures, we induced *A. brasilense* cells for spirilobactin production by growth in low-iron medium at 32°C, split the cultures into two flasks, and allowed growth in one flask to continue at 32°C while the temperature in the other flask was shifted to 42°C (Fig. 3). In the control cultures grown at 32°C, spirilobactin production increased along with growth until it reached a maximum value of 15 nmol of dihydroxybenzoic acid per ml of medium per unit cell optical density at 590 nm (OD_{590}) and remained constant thereafter, indicating that these cells were continuously synthesizing spirilobactin at a constant rate. In the cultures grown where the temperature was shifted to 42°C, spirilobactin production continued further for 3 h (approximately 1 doubling time), after which the production eased completely although growth continued unaltered. This temperature shift experiment clearly indicates a temperature-dependent step or regulatory element in the biosynthesis of spirilobactin. In addition, the experiment also showed the ability of *A. brasilense* to grow continuously at 42°C in low-iron medium (measured up to approximately 4.0 OD_{590}) when spirilobactin was available; in contrast, growth of the bacteria stopped completely in low-iron medium at 42°C at about approximately 1.6 OD_{590} where spirilobactin production was not possible (Fig. 1).

To examine the ability of *A. brasilense* to transport iron complexed to spirilobactin at 42°C, cells were induced for the transport system at 32°C and transport was measured at different temperatures as described earlier (3). Transport activities at 32 and 42°C were found to be comparable, although the optimum temperature for transport was 35°C (Fig. 4).

We had earlier shown the induction of four high-molecular-weight (87K [87,000], 83K, 78K, and 72K) outer membrane proteins under conditions of iron limitation (2). Since spirilobactin production was inhibited at 42°C, it was of

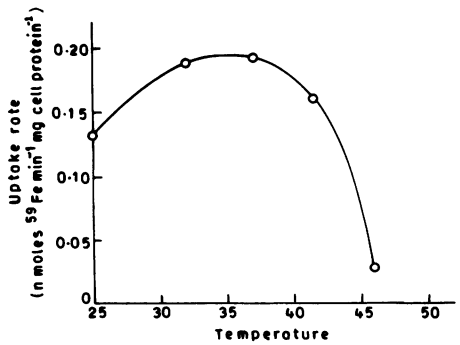

FIG. 4. Effect of temperature on uptake of ^{59}Fe from ^{59}Fe-spirilobactin complex. Induced cells and ^{59}Fe-spirilobactin complex solutions were preincubated at the uptake temperature for 30 min for temperature equilibration, and uptake experiments were performed at the indicated temperature as described in the text.

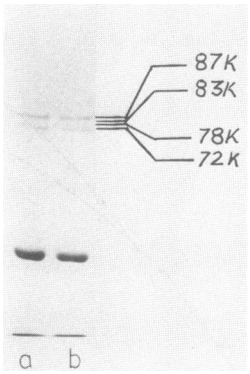

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% [wt/vol] acrylamride) showing the induction of iron-regulated outer membrane protein of *A. brasilense* RG growing at 32 (lane a) and 42°C (lane b). Ethylenediamine-di(*o*-hydroxyphenylacetic acid) (50 μg/ml) was added to exponentially growing cells in Luria broth-succinate medium and harvested after 3 h, and outer membrane proteins (indicated by their molecular weights) were isolated as described in the text.

interest to see whether these proteins were also affected. However, we observed no significant inhibition of the induction of production of these proteins when cells were grown at 42°C, compared with cells grown at 32°C (Fig. 5).

The results described here demonstrate that apart from its regulation by iron, siderophore production in *A. brasilense* was inhibited 20-fold by growth at higher temperatures. Furthermore, despite this very strong inhibition of siderophore production by temperature, the other components of the siderophore transport system, namely, the induction of the iron-regulated outer membrane receptor proteins and the transport of ferric spirilobactin, were not significantly affected by growth at these temperatures. The need for this additional temperature-dependent regulatory element in siderophore biosynthesis alone is unclear at this stage.

Our results constitute the first observation of temperature inhibition of siderophore production in a nitrogen-fixing microorganism and is of particular importance as this tropically located nitrogen-fixing soil bacterium cannot fix nitrogen at temperatures above 40°C (5). The possibility of a linkage in the regulatory elements of these two phenomena, however, awaits more detailed investigations.

A.K.B. was supported by a fellowship from the Council of Scientific and Industrial Research, India.

LITERATURE CITED

1. **Arnow, L. E.** 1937. Colorimetric determinations of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118**:531–537.
2. **Bachhawat, A. K., and S. Ghosh.** 1987. Isolation and characterisation of the outer membrane proteins of *Azospirillum brasilense*. J. Gen. Microbiol. **133**:1751–1758.
3. **Bachhawat, A. K., and S. Ghosh.** 1987. Iron transport in *Azospirillium brasilense*: role of the siderophore, spirilobactin. J. Gen. Microbiol. **133**:1759–1765.
4. **Cogswell, R. L., and E. D. Wienberg.** 1980. Temperature restriction of iron acquisition in *Proteus vulgaris*. Microbios Lett. **15**:69–71.
5. **Day, J. M., and J. Dobereiner.** 1976. Physiological aspects of nitrogen fixation by *Spirillum* from *Digitaria* roots. Soil Biol. Biochem. **8**:45–50.
6. **Elmerich, C.** 1984. Molecular biology and ecology of dizotrophs associated with non-leguminous plants. Bio/Technology **2**:967–978.
7. **Fekete, F. A., J. T. Spence, and T. Emery.** 1983. Siderophores produced by nitrogen-fixing *Azotobacter vinelandii* OP in iron-limited continuous cultures. Appl. Environ. Microbiol. **46**: 1297–1300.
8. **Garibaldi, J. A.** 1971. Influence of temperature on the iron metabolism of a fluorescent pseudomonad. J. Bacteriol. **105**: 1036–1038.
9. **Ismail, A., G. W. Bedell, and D. M. Lupan.** 1985. Effect of temperature on siderophore production by *Candida albicans*. Biochem. Biophys. Res. Commun. **132**:1160–1165.
10. **Kluger, M. J., and B. A. Rothenberg.** 1979. Fever and reduced iron: their interaction as a host defense response for bacterial infection. Science **203**:374–376.
11. **Maulik, P., and S. Ghosh.** 1986. NADPH/NADH-dependent cold labile glutamate dehydrogenase in *Azospirillum brasilense*. Purification and properties. Eur. J. Biochem. **155**:595–602.
12. **Van Berkum, P., and B. B. Bohlool.** 1980. Evaluation of nitrogen fixation by bacteria in association with roots of tropical grasses. Microbiol. Rev. **44**:491–517.
13. **Worsham, P., and J. Konisky.** 1984. Effect of growth temperature on the acquisition of iron by *Salmonella typhimurium* and *Escherichia coli*. J. Bacteriol. **158**:163–168.