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Colonial Heterogeneity of *Thiobacillus versutus*

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In acetate-limited chemostat cultures started with single-colony cultures of *Thiobacillus versutus*, a mutant appeared after approximately 85 volume changes. The inhomogeneity of the culture was detected by the development of two different types of colonies on agar plates. When a pure culture of the mutant was grown in a chemostat, parent colonies appeared after almost the same period of time. Electron micrographs of the mutant grown on butyrate showed the presence of fibrils surrounding the cells. The cells of the parent strain were bald when grown under the same conditions. The growth kinetics of the parent and the mutant were investigated in batch cultures with a variety of substrates and were found to be identical. Major differences between the two strains were observed during growth on mannitol; the mutant attained a lower yield and excreted large amounts of extracellular polysaccharides.

The loss of homogeneity of a bacterial culture during cultivation in a rigidly controlled environment is not an unknown phenomenon. Calcott (3) recently summarized a number of studies in which parent-mutant transitions in chemostat cultures were observed. In continuous cultures, at nutrient concentrations much lower than the K_s, the outcome of the competition between parent and mutant depends on the μ_{max}/K_s ratio. At higher substrate concentrations, the competition is always won by the organism with the lowest K_s, except for the situation in which the Monod curves cross over. In this situation, at substrate concentrations below the crossover point, K_s is decisive, but at higher concentrations the organism with the highest growth rate is selected (16). Physiological causes of parent-mutant systems have been investigated in a number of cases (3). Although some of these studies have led to an understanding of the observed phenomenon, in some transitions take-over by a mutant without obvious reasons has been noticed.

There are two possible ways to cope with the problem of the appearance of mutants in a pure culture. The first is to maintain a chemostat culture only for a short period of time, as suggested by Tempest (14); the second one is to wait until the most adapted organism has established itself (8).

Here we report the appearance of a mutant with a different colonial morphology in a pure chemostat culture of *Thiobacillus versutus*. To identify the nature of this parent-mutant transition, some growth kinetic and physiological parameters of both strains have been investigated in chemostat and batch cultures.

MATERIALS AND METHODS

Organism, media, and cultivation. A *T. versutus* strain formerly called A2 (ATCC 25364) (5) was a gift from J. G. Kuenen (Delft, The Netherlands); in this paper this strain is called WS. The organism was maintained on thiosulfate agar slants stored at 4°C and subcultured every 2 months. The basal medium for growth in batch culture was that of Taylor and Hoare (13), containing 2 ml instead of 5 ml of trace element solution as described by Gottschal and Kuenen (4). For growth on methanol, formate, methylamine, ethanol, glutamate, 5-aminovalerate, and organic acids, substrates were added at a concentration of 2.5 g of the sodium salt per liter (except for methanol and ethanol). In the medium with methylamine, NH_4Cl was omitted. For growth in batch culture on mannitol, 1.1 g of sodium glutamate per liter was substituted for NH_4Cl. Mannitol, sterilized separately, was added to a final concentration of 5.0 g/liter. Growth on mannitol was also studied in a medium described by Zevenhuizen and van Neerven (19). Solid media were prepared by adding 15 g of agar (GIBCO, Europe Ltd., Paisley, Scotland) per liter. The medium for chemostat cultures was that of Gottschal and Kuenen (4). Sodium acetate was added at a final concentration of 5.0 g/liter. Cultures were grown at a dilution rate of 0.1 h^{-1}. The pH was kept at 8.0 through automatic addition of 1 N HCl. Oxygen was supplied by sparging air through the culture at a rate of 1 liter of air per liter of culture per min. All cultures were grown at 30°C.

Colony transformation. Samples from the chemostat culture were regularly streaked onto agar plates with batch culture medium. Photographs were taken with a Wild M5 microscope with the enlargement set at 12×. The mutant proportion was estimated by spreading 0.1 ml of a dilution series onto agar plates with the same substrate as used in the chemostat.

Electron microscopy. Butyrate-grown cells were washed with 0.1 M cacodylate buffer (pH 7.2). Pellets were suspended in 1% (wt/vol) purified agar (GIBCO) in cacodylate buffer at 45°C, followed by fixation in 2% (wt/vol) paraformaldehyde and 2.5% (wt/vol) glutaraldehyde in the same buffer. After fixation in 1% (wt/vol) OsO_4, the specimens were dehydrated in graded ethanol and placed in propylene oxide. Dehydrated samples were embedded in Epon 812. Thin sections were stained with uranyl acetate (30 min at 40°C) and lead citrate (40 s at 20°C) with a LKB Bromma 2168 Ultrostainer.

Analytical methods. Growth in batch culture was followed by measuring the optical density of the bacterial suspension at 623 nm with a Vitatron Universal Photometer. The specific growth rate was calculated from the slope of the linear interval of the logarithmic growth curve. The dry weight of samples from the cultures was determined by washing the cell pellet from 50 ml of culture twice with distilled water. The washed cell pellet was dried overnight at 105°C in predried and preweighed vials. Dissolved acetate in the chemostat culture was analyzed with a Varian gas

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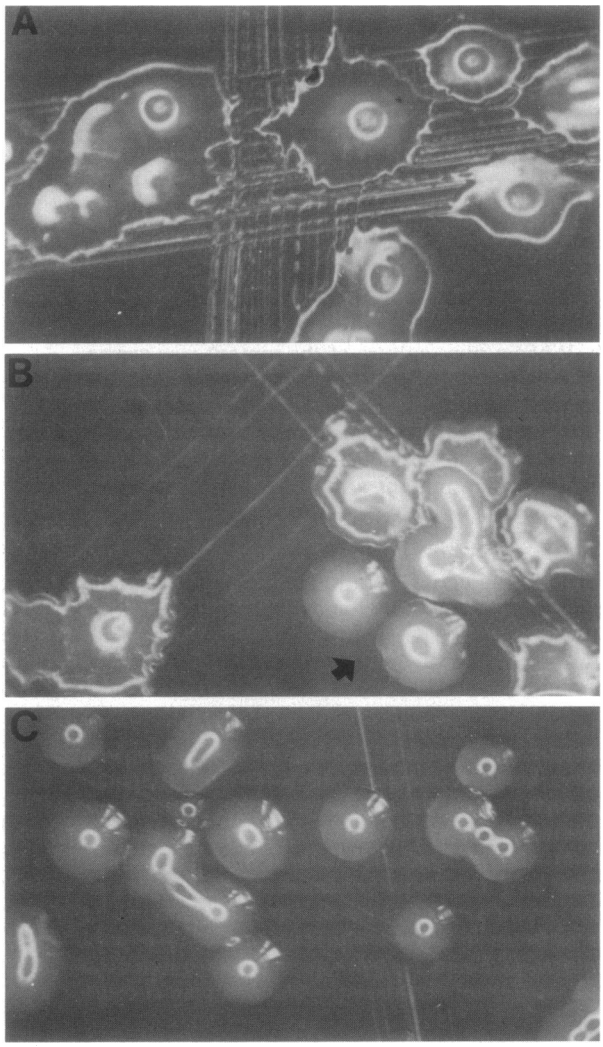

FIG. 1. Growth of *T. versutus* WS and WH on agar plates containing acetate as the carbon and energy source. A, Sample from a pure batch culture of WS on acetate used to inoculate the chemostat. B, Sample from the chemostat culture on acetate after approximately 85 volume changes. The arrow indicates WH colonies. C, Sample from a pure batch culture of WH on acetate.

chromatograph equipped with a Chromosorb 101, 80-100-mesh, column. The total carbohydrate content of batch-grown cells harvested through centrifugation was determined with the anthrone-sulfuric acid reagent with glucose as a standard (15). The amount of poly-3-hydroxybutyric acid was measured in 96% H_2SO_4 at 235 nm after extraction of acid-hydrolyzed cells with chloroform (18). Extracellular sugars in the supernatant of centrifuged cultures were analyzed with the anthrone-sulfuric acid reagent (15) by the method of Somogyi (10) for the determination of reducing end groups. Mannitol concentrations were spectrophotometrically quantified after oxidation with periodate followed by the chromotropic acid-formaldehyde reaction (2). For a qualitative determination mannitol was acetylated with acetic anhydride by using 1-methylimidazole as the catalyst (1). The alditol acetate was extracted into dichloromethane and subsequently identified by gas chromatography with a Kipp Analytica 8200 gas chromatograph equipped with a WCOT fused Silica OV 225 capillary column (length, 25 m) at 210°C. Identification was done by comparison with the derivative of a pure solution of mannitol.

RESULTS

The original *T. versutus* strain, WS, forms colonies which are best described as having a rather dense central region surrounded by a more or less irregularly shaped, spreading fringe (Fig. 1A). These colonies fit the description of Taylor and Hoare (13). After continuous cultivation of this strain in a chemostat for approximately 3 weeks (approximately 85 volume replacements), a novel type of colonies having smooth edges appeared besides the original when samples were streaked onto agar plates (Fig. 1B and 1C). Bacteria which form this type of colony have subsequently been called *T. versutus* WH. Continuous cultivation of a pure culture of WH led to the appearance of colonies resembling WS colonies. When observed through a light microscope the cells of each strain are indistinguishable: WS and WH cells are coccobacillary in shape. Electron micrographs of *T. versutus* WS and WH, on the other hand, demonstrate an important difference in the cell surface (Fig. 2); WS cells are bald, and WH cells are surrounded by fibrils.

The yield of *T. versutus* WS and WH in a chemostat culture on acetate was, respectively, 0.36 and 0.40 g/g of acetate. Thus only a small difference could be observed. The ratio of the WH mutants in a WS culture or vice versa varied between 1 and 10 per 1,000 cells after approximately 85 volume changes on acetate. After 6 weeks in the chemostat (approximately 170 volume changes) this ratio did not change. Hence the observed mutant proportion seemed to have reached an equilibrium and was definitely higher than the ratio observed as a result of spontaneous mutations.

The versatility of *T. versutus* WS and WH with respect to growth potential was studied with a variety of substrates. Both strains grew in mineral media supplemented with the following substrates: thiosulfate, formate, methylamine, ac-

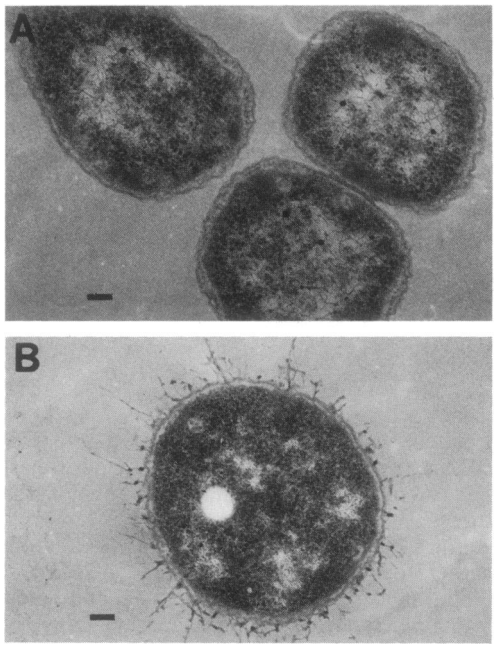

FIG. 2. Ultrathin sections of *T. versutus* grown on butyrate. A, Strain WS, with a bald cell surface. B, Strain WH, with fibrils surrounding the cell wall. Bars, 0.1 μm.

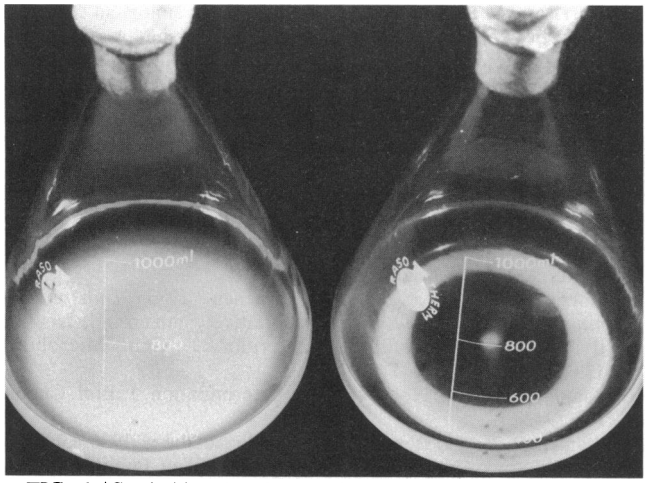

FIG. 3. Settled batch cultures of *T. versutus* WS (right) and WH (left) after growth on mannitol. The medium was that of Zevenhuizen and van Neerven (19). Note the diffuse layer on top of the WH pellet.

etate, ethanol, glycolate, lactate, butyrate, 4-hydroxybutyrate, succinate, malate, 2-oxoglutarate, glutamate, glutaconate, adipate, 5-aminovalerate, and mannitol. During growth on formate both strains formed clumps. Neither strain grew on methanol, not even after 2 weeks of incubation at 30°C. Growth on 4-hydroxybutyrate was characterized by an extremely long lag phase of 9 days. A selected number of maximum specific growth rates (μ_{max}) of *T. versutus* WS and WH in batch culture were determined in media supplemented with the following substrates: thiosulfate, acetate, succinate, glutarate, glutamate, and mannitol. The values for the μ_{max} were virtually the same and ranged from 0.043/h (WS) and 0.046/h (WH) for growth on thiosulfate to 0.45/h (WS) and 0.44/h (WH) on succinate. Growth in basal medium supplemented with mannitol as the carbon and energy source and glutamate as the nitrogen source yielded a remarkable difference between the strains. The optical density at 623 nm of stationary-phase WS cells was about 1.8 times higher than that of stationary-phase WH cells. On the contrary, the values for μ_{max} were almost identical, 0.30/h (WS) and 0.32/h (WH).

When *T. versutus* WS and WH were grown on mannitol and glutamate in a different medium, composed as described by Zevenhuizen and van Neerven (19), an even higher optical density was obtained with both strains. The previously observed difference in maximal optical density of WS and WH cultures was again noticed, together with the similarity in μ_{max}:0.29/h for WS and WH. The medium of Zevenhuizen and van Neerven (19) contained less phosphate, more Ca and Zn ions, but fewer Mn, Mo, Cu, Co, and Fe ions than the medium used before. Furthermore, this medium was supplemented with the B vitamins biotin and thiamine. As omission of the vitamins was without effect, the high optical densities of the cultures after growth in the Zevenhuizen-van Neerven medium were attributed to differences in mineral composition of the medium used. When cultures of *T. versutus* WS and WH grown on mannitol in the Zevenhuizen-van Neerven medium were left to settle, an interesting phenomenon was observed. *T. versutus* WS developed a compact pellet with a clear supernatant, whereas WH developed a pellet on top of which a thick layer of a diffuse substance was noticed. The supernatant was again very clear (Fig. 3). The cells of both cultures had the normal coccobacillary shape of *T. versutus* and appeared to be stuffed with poly-3-hydroxy-butyrate. Lysed cells were not visible. The macroscopic differences between WS and WH cells after growth on mannitol and glutamate were investigated by chemical analysis of the cultures (Table 1). The composition of the cells of WS and WH was roughly the same. In the extracellular fluid, however, distinct differences were found. The yield of strain WS was 0.5 g/g of substrate, whereas WH reached only 0.4 g/g. The amount of mannitol left in the supernatant of the WH culture, quantified spectrophotometrically and identified by gas chromatographic analysis, was considerable. Furthermore, strain WH produced a large amount of extracellular polysaccharides with a composition different to the polysaccharides excreted by *T. versutus* WS. The production of the extracellular material had apparently proceeded at the cost of biomass production.

DISCUSSION

The appearance of a novel colony type in a culture which was homogeneous with respect to colony shape is called dissociation (11). The most well-known change is the transformation of smooth into rough colonies by *Brucella abortus*. At first this seemed to be a reversible mutation, but later it became clear that the new smooth cells differed from the originals in a distinct physiological aspect. Replacement of a parent organism by a variant with a different colony shape has also been observed in chemostat cultures of *Enterobacter aerogenes*. This transition was accompanied by changes in biochemical and immunological composition, in polysaccharide content, in survival properties, and in substrate-accelerated death (12). Despite the physiological differences between parent and mutant in these studies, morphological transitions without any effect on growth have also been reported (11). *T. versutus* WS and WH seem to belong to this last category, since the growth-kinetics of both strains were identical (see above). However, a marked

TABLE 1. Parameters of growth of *T. versutus* WS and WH on mannitol[a]

Strain	Incubation time (h)	Pellet			Supernatant		
		Dry wt (μg/ml)	% Carbohydrate	% Poly-3-hydroxy-butyrate	Mannitol (μg/ml)	Extracellular polysaccharides (μg/ml)	% Reducing sugars
WS	48	3,006	30	40	0	343	9
	72	3,006	12	58	0	289	10
WH	48	1,919	11	40	937	1,914	74
	72	2,025	16	38	957	1,829	86

[a] Cells were harvested after 48 or 72 h of incubation at 30°C. The concentration of the substrate (mannitol) was 5.0 g/liter. The medium was that of Zevenhuizen and van Neerven (19).

difference was observed in the ultrastructure of WS and WH cells after growth on butyrate (Fig. 2) and in the composition of excreted polysaccharides during growth on mannitol (Table 1). At this moment we are not sure whether these strain differences are intrinsic to the strain or to the substrate. However, the observed differences illustrate the phenomenon that mutants appearing during prolonged continuous cultivation on acetate can differ in growth on other substrates as well. Even though we were able to determine two major strain differences on arbitrarily chosen substrates, we are still confused about the real nature of the transition, since apparently (i) during growth of *T. versutus* on acetate mutants arise easily, (ii) the mutation is reversible, and (iii) no selective advantage as far as growth on acetate is concerned can be detected. Previous reports on growth of *T. versutus* on methanol (6, 13; J. P. van Dijken and J. G. Kuenen, Abstr. Third Int. Symp. Microbial Growth on C_1 Compounds, p. 36–37, 1984), its capacity to oxidize thiosulfate under denitrifying conditions (9, 13, 17), and its motility (7, 13) are controversial. In view of the here reported ease of mutation we feel that the occurrence of strain differences should be taken into account.

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LITERATURE CITED

1. **Blakeney, A. B., P. J. Harris, R. J. Henry, and B. A. Stone.** 1983. A simple and rapid preparation of alditol acetates for mono-saccharide analysis. Carbohydr. Res. **113**:291–299.
2. **Burton, R. M.** 1957. The determination of glycerol and dihydroxyacetone. Methods Enzymol. **3**:246–249.
3. **Calcott, P. H.** 1981. Genetic studies using continuous culture, p. 127–140. *In* P. H. Calcott (ed.), Continuous cultures of cells, vol. 2. CRC Press, Boca Raton, Fla.
4. **Gottschal, J. C., and J. G. Kuenen.** 1980. Mixotrophic growth of *Thiobacillus* A2 on acetate and thiosulfate as growth limiting substrates in the chemostat. Arch. Microbiol. **126**:33–42.
5. **Harrison, A. P., Jr.** 1983. Genomic and physiological comparisons between heterotrophic Thiobacilli and *Acidiphilium cryptum*, *Thiobacillus versutus* sp. nov., and *Thiobacillus acidophilus* nom. rev. Int. J. Syst. Bacteriol. **33**:211–217.
6. **Kelly, D. P., and A. P. Wood.** 1982. Autotrophic growth of *Thiobacillus* A2 on methanol. FEMS Microbiol. Lett. **15**:229–233.
7. **Korhonen, T. K., E.-L. Nurmiaho, and O. H. Tuovinen.** 1978. Fimbriation in *Thiobacillus* A2. FEMS Microbiol. Lett. **3**:195–198.
8. **Postgate, J. R., and J. R. Hunter.** 1962. The survival of starved bacteria. J. Gen. Microbiol. **29**:233–264.
9. **Robertson, L. A., and J. G. Kuenen.** 1983. *Thiosphaera pantotropha* gen. nov. sp., a facultatively anaerobic, facultatively autotrophic sulphur bacterium. J. Gen. Microbiol. **129**:2847–2855.
10. **Somogyi, M.** 1952. Notes on sugar determination. J. Biol. Chem. **195**:19–23.
11. **Stanier, R. Y., M. Doudoroff, and E. A. Adelberg.** 1966. General microbiology, 2nd ed., p. 467–488. Macmillan Publishing Co., Inc., London.
12. **Strange, R. E., and J. R. Hunter.** 1966 "Substrate-accelerated" death of nitrogen-limited bacteria. J. Gen. Microbiol. **44**:255–262.
13. **Taylor, B. F., and D. S. Hoare.** 1969. New facultative *Thiobacillus* and a reevaluation of the heterotrophic potential of *Thiobacillus novellus*. J. Bacteriol. **100**:487–497.
14. **Tempest, D. W.** 1970. The place of continuous culture in microbiological research. Adv. Microb. Physiol. **4**:223–250.
15. **Trevelyan, W. E., and J. S. Harrison.** 1952. Studies on yeast metabolism. I. Fractionation and microdetermination of cell carboydrates. Biochemistry **750**:298–310.
16. **Veldkamp, H., H. van Gemerden, W. Harder, and H. J. Laanbroek.** 1984. Microbial competition, p. 279–290. *In* M. J. Klug and C. A. Reddy (ed.), Current perspectives in microbial ecology. American Society for Microbiology, Washington, D.C.
17. **Wood, A. P., and D. P. Kelly.** 1983. Autotrophic, mixotrophic and heterotrophic growth with denitrification by *Thiobacillus* A2 under anaerobic conditions. FEMS Microbiol. Lett. **16**:363–370.
18. **Zevenhuizen, L. P. T. M.** 1981. Cellular glycogen, β-1,2-glucan, poly-β-hydroxybutyric acid and extracellular polysaccharides in fast-growing species of *Rhizobium*. Antonie van Leeuwenhoek. J. Microbiol. Serol. **47**:481–497.
19. **Zevenhuizen, L. P. T. M., and A. R. W. van Neerven.** 1983. (1→2)-β-D-glucan and acidic oligosaccharides produced by *Rhizobium meliloti*. Carbohydr. Res. **118**:127–134.