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# Dependence of Proliferation of *Bacteroides forsythus* on Exogenous *N*-Acetylmuramic Acid

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***Bacteroides forsythus* is the first wild-type or mutant bacterium found to require exogenous *N*-acetylmuramic acid for proliferation and maintenance of cell shape. This implies so far unknown pathways for peptidoglycan synthesis and a strict dependence of *B. forsythus* on other bacteria in its oral habitat. Addition of *N*-acetylmuramic acid to conventional bacteriological media allows routine cultivation of this fastidious organism.**

Changes in the composition of the highly complex mixed populations of bacteria in gingival pockets occur concurrent to the development of periodontal diseases. To document such changes and find correlations with disease development, specific members of the flora should be quantitatively monitored. However, many of the oral bacteria have special nutritional demands and thus are difficult to detect by available culture methods (11). Advances in the understanding of the metabolism of such fastidious bacteria may be expected to lead to improved methods for their cultural detection. To this end, the chemically defined tissue culture medium ZW (15) has been modified to make it adequate for the recovery of many oral bacteria, including the fastidious *Wolinella* and *Campylobacter* group organisms (16). Some microorganisms, however, proved to be more demanding: black-pigmenting *Bacteroides* could only grow when this defined medium was supplemented with fetal calf serum (FCS) (16). Even more demanding were *Treponema vincentii* and *Bacteroides forsythus*, which in addition to FCS needed further growth-promoting components. For *T. vincentii*, one such growth factor, isolated from milk, was identified as *N*-acetylglucosamine-1-phosphate (2).

The present study on growth factors for *B. forsythus* demonstrates a unique requirement of this organism for *N*-acetylmuramic acid (MurNac). This may explain previous difficulties in culturing *B. forsythus*.

## MATERIALS AND METHODS

**Bacterial strains.** The origins of all bacterial strains used in this study have been described before (7, 14).

**Media and growth conditions.** The synthetic tissue culture medium ZW (15) was modified and prepared as described for the *Wolinella* and *Campylobacter* group (16) but without sodium formate, sodium fumarate, and potassium nitrate (2). The origins of the chemically complex medium ingredients were as follows: Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), Actinomyces broth (Difco Laboratories, Detroit, Mich.), thioglycolate medium (Difco), brain-heart infusion broth (BHI; Difco), Noble agar (Difco), Columbia blood agar base (Difco), and FCS (Flow Laboratories, Inc., McLean, Va.). All cultures were incubated anaerobically (GasPak System; BBL) at 36°C for up to 7 days. For semiquantitative tests, supplements were dissolved in water, adjusted to pH 7, filter sterilized, and added in volumes of up to 0.1 ml to duplicate 1-ml cultures in 24-well multidishes (Nunc, Roskilde, Denmark). For quantitative tests, triplicate 20-ml cultures were set up in 30-ml polystyrene tubes.

**Chemicals.** All chemicals required for the modified ZW (M/ZW) medium or tested as supplements were of the highest available purity (E. Merck AG, Darmstadt, Federal Republic of Germany [FRG]; Sigma Chemical Co., St. Louis, Mo.; and Serva, Heidelberg, FRG).

## RESULTS AND DISCUSSION

**Screening for growth factors.** Three strains of *B. forsythus*, OMZ 408, FDC 331, and ATCC 43037, in M/ZW with 5% FCS were used to screen numerous compounds in concentrations of up to 1 g/liter for possible growth-promoting activity. With the exception of MurNac, all compounds tested failed to promote growth (Table 1). Only the addition of MurNac to the medium allowed proliferation of the three strains of *B. forsythus*. At 0.1 mg/liter, the minimal effective concentration of MurNac, cell concentrations remained low. Also, a large proportion of the cells developed into spherical forms, indicative of defective cell wall structure. However, when MurNac was used at at least 1 mg/liter, the cell population of each strain was morphologically homogeneous and densities exceeded $10^7$ cells per ml.

All three strains of *B. forsythus* have been kept for more than 20 passages (1:1,000 dilution each) in M/ZW with 5% FCS and MurNac (10 mg/liter), and no evidence of a loss of MurNac dependence or proliferation potential has been detected.

These results demonstrate that MurNac is an essential nutrient for *B. forsythus* and cannot be replaced by related compounds. This is in marked contrast to the results with *T. vincentii*, for which MurNac proved to act as a growth factor but could be substituted for by a number of other N-acetylated carbohydrates (Table 1). Indeed, no other wild-type or mutant bacterium has so far been reported to have this highly specific requirement for MurNac. Since this compound is not known to be synthesized by the human host, *B. forsythus* appears to be strictly dependent on other bacteria. This is consistent with reports that proliferation of *B. forsythus* was stimulated in vitro by metabolites of a wide range of gram-positive and gram-negative oral bacteria (4, 12, 14). It may be noted, however, that some metabolites of *Fusobacterium nucleatum* FDC 364 with growth-promoting activity in the present assay system have been found to be

TABLE 1. Ability of various compounds to promote proliferation of *B. forsythus* and *T. vincentii*[a]

| Compound added | Proliferation | |
|---|---|---|
| | *B. forsythus* | *T. vincentii* |
| Solvent control | − | − |
| MurNac | + | + |
| Muramic acid | − | − |
| *N*-Acetylglucosamine | − | + |
| *N*-Acetylgalactosamine | − | − |
| *N*-Acetylglucosamine-1-phosphate | − | + |
| *N*-Acetylglucosamine-6-phosphate | − | + |
| *N*-Acetylgalactosamine-1-phosphate | − | − |
| UDP-*N*-acetylglucosamine | − | + |
| Phosphoenolpyruvate | − | − |
| *N*-Acetylmannosamine | − | − |
| *N*-Acetyllactosamine | − | − |
| Chitobiose | − | + |
| Chitotetrose | − | + |
| *N*-Acetylneuraminic acid | − | − |
| Mucin (porcine submaxillary) | − | + |

[a] Proliferation of three strains of *B. forsythus* and one of *T. vincentii* was tested in M/ZW medium with 5% FCS. Compounds tested were added at concentrations of up to 1 g/liter.

chromatographically distinct from MurNac (data not shown). They are likely to contain MurNac and are probably muramylpeptides, which are known to be potent modulators of immunoreactivity (1) and bone metabolism (3). Proliferating *B. forsythus* may thus remove free muramylpeptides from the subgingival space, and thereby at least indirectly influence disease development.

**Salvage of MurNac.** The synthesis of MurNac and its extension to muramylpeptides has so far only been known to occur on a uridine diphosphate (UDP) carrier (8). The above results, however, imply the activity of a salvage pathway for preformed MurNac. To search for a similar activity in other bacteria, phosphomycin was used to block endogenous UDP-MurNac synthesis (9). With this treatment, phosphomycin-sensitive cells can only be expected to grow if they are able to take up and incorporate exogenous MurNac. However, the presence of MurNac (30 mg/liter) in M/ZW with 5% FCS did not abolish the sensitivity to phosphomycin of *Escherichia coli* ATCC 25922, *Wolinella recta* FDC 267R, and *F. nucleatum* FDC 364. The MICs of phosphomycin for these three strains, irrespective of the presence of absence of MurNac, were 0.01, 0.03, and 0.001 mg/ml, respectively. The three (MurNac-dependent) strains of *B. forsythus* were resistant to phosphomycin at concentrations up to 1 mg/ml. These data indicate that the ability to salvage exogenous MurNac for peptidoglycan synthesis may be unique to *B. forsythus*. This should make that organism useful for studies of sacculus synthesis and turnover (8).

**Factors limiting the growth of *B. forsythus* in complex medium.** The dependence of *B. forsythus* on exogenous MurNac was first found in a synthetic medium. Such defined media are obviously desirable for analytical studies. However, bacteriological routine favors simpler techniques. To determine whether a lack of available MurNac in conventional media could explain previous difficulties in culturing *B. forsythus* (4–6, 10, 13, 14), commercial broth media were supplemented with 5% FCS or MurNac (10 mg/liter) or both. In these tests, Trypticase soy broth and Actinomyces broth did not support proliferation of any of the three *B. forsythus* strains. The results with fluid thioglycolate medium and BHI broth are summarized in Table 2. The data clearly demonstrate that proliferation of all three strains of *B. forsythus* was limited by the availability of exogenous MurNac as well as some unidentified component(s) of FCS.

TABLE 2. Growth of three strains of *B. forsythus* in M/ZW and two complex media[a]

| Medium and strain | Growth | | | |
|---|---|---|---|---|
| | No FCS | | With FCS | |
| | No MurNac | With MurNac | No MurNac | With MurNac |
| M/ZW | | | | |
| FDC 331 | − | − | − | ++ |
| ATCC 43037 | − | − | − | ++ |
| OMZ 408 | − | − | − | ++ |
| Thioglycolate | | | | |
| FDC 331 | − | − | − | +++ |
| ATCC 43037 | − | − | − | − |
| OMZ 408 | − | − | + | + |
| BHI broth | | | | |
| FDC 331 | − | − | − | +++ |
| ATCC 43037 | − | − | − | +++ |
| OMZ 408 | − | +++ | − | +++ |

[a] Media were supplemented with 5% FCS, MurNac (10 mg/liter), or both after 5 days of incubation. Growth was graded visually on a scale from no growth (−) to very dense growth (+++).

The quantitative relationship between the initial concentration of MurNac and the cell densities obtained with *B. forsythus* strains FDC 331 and ATCC 43037 in 5% FCS-supplemented BHI is shown in Fig. 1. A pronounced growth-promoting effect was seen with 1 μM MurNac (0.3 mg/liter). Maximum cell densities of more than $2 \times 10^8$ cells per ml, or about 5 mg of cell wet weight per ml, were observed with 0.1 mM MurNac (30 mg/liter).

Finally, in experiments with commercial agar media (BHI and Columbia blood agar base), all three strains of *B. forsythus*, incubated for 6 days, were found to form convex colonies up to 1 mm in diameter when the media were

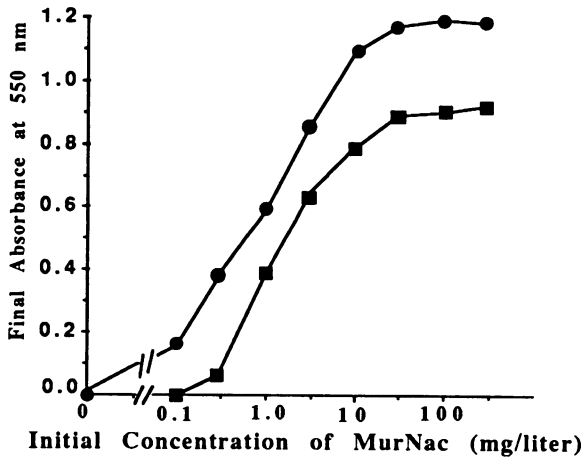


FIG. 1. Dependence of *B. forsythus* proliferation on the concentration of exogenous MurNac. Strains FDC 331 (●) and ATCC 43037 (■) were grown anaerobically for 5 days in BHI broth with 5% FCS and the indicated concentrations of MurNac. Means of $A_{550}$ values for triplicate cultures were plotted (variation was <10%).

supplemented with MurNac (10 mg/liter) and either 5% FCS or 5% laked human blood. Thus, the tedious and contamination-prone use of feeder strains or conditioned media otherwise needed to foster *B. forsythus* proliferation (4, 5, 10, 13, 14) could be eliminated, with a simultaneous increase in yield. This improved method has recently allowed the efficient cultural monitoring of *B. forsythus* in experimentally infected rats (B. Guggenheim, R. Gmür, R. Schmid, and C. Wyss, Am. Assoc. Dent. Res. Annu. Session 1989, J. Dent. Res., vol. 68, abstr no. 1691).

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