INFECTION AND IMMUNITY, Aug. 1983, p. 516–522
0019-9567/83/080516-07$02.00/0

Vol. 41, No. 2

Modulation of *Bordetella pertussis* by Nicotinic Acid

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Received 22 February 1983/Accepted 18 May 1983

Growth of *Bordetella pertussis* in a high concentration of nicotinic acid (NA) had a modulating effect on several properties and activities of the bacteria. Compared with normally grown cells, those grown in a high concentration of NA had reduced capacity for taking up both NA and nicotinamide (ND); they had reduced adenylate cyclase activity and showed loss of agglutinogen factors 2 and 3, but an increase in factor 1. By contrast, cells grown in a high concentration of ND showed only a slightly decreased capacity for uptake of ND and none of the other changes. Modulation of *B. pertussis* by NA varied with the strain and culture conditions and appeared to be distinct from the antigenic modulation induced by high Mg^{2+} in the culture medium. Evidence is presented for the association of a small proportion of the extracytoplasmic adenylate cyclase with the outer membrane of *B. pertussis*.

Bordetella pertussis, the causative organism of whooping cough, shows several types of reversible, phenotypic loss of cellular components in response to growth conditions.

The first of these to be described, "antigenic modulation" (21, 21a), is induced either by changing the ionic composition of the medium (typically by replacing sodium chloride with magnesium sulfate) or by lowering the temperature of growth from 35 to 28°C. Properties lost by antigenic modulation include cell envelope components such as pertussigen (18, 25, 33, 34), agglutinogens (2), cytochrome d_{629} (9), several envelope bands seen in polyacrylamide gels (9, 34), the cytoplasmic heat-labile toxin (8, 22), and the predominantly extracytoplasmic adenylate cyclase (AC) (14, 28).

A second form of reversible phenotypic variation, known as "nicotinic acid modulation" (29), is induced by increasing the nicotinic acid (NA) concentration in the growth medium 100-fold from 5 to 500 μg/ml. Although not studied as extensively as antigenic modulation, NA modulation is known to result in a similar loss of properties, namely pertussigen (1, 29, 34) and the same main cell envelope proteins. However, there was no loss of heat-labile toxin (29). This, together with a different serological response, led Pusztai and Jóo (29) to suggest that antigenic modulation and NA modulation were different.

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B. pertussis has an absolute nutritional requirement for either NA or nicotinamide (ND), both being equally effective in supporting growth (17, 19). Wardlaw et al. (34), however, reported that growth in a high concentration of ND, unlike that in NA, had no effect on the pertussigen or main envelope proteins of *B. pertussis*.

Growth in high concentrations of vitamins is known to repress vitamin transport systems in a variety of bacteria (reviewed by Kadner [20]). In previous reports, the characteristics of uptake of NA and ND by *B. pertussis* grown in low concentrations of NA were described (23, 24) and found to be very similar.

We report here on the effect of growth of *B. pertussis* in high concentrations of NA or ND on the uptake of those vitamins, on the heat-labile agglutinogens of the cell envelope, and on the extracytoplasmic AC.

MATERIALS AND METHODS

Chemicals. ^{14}C-labeled NA and ND were obtained from Amersham Corp. Unlabeled NA and ND, EDTA, and Formalin (37 to 39% formaldehyde solution) were obtained from BDH. Tris, *N*-Tris-(hydroxymethyl)-methyl glycine (Tricine), ATP, creatine phosphate, and creatine phosphokinase were all purchased from Sigma Chemical Co. Where available, chemicals were of analytical grade.

Strains and growth conditions. *B. pertussis* 134 and 18323 were from the culture collection of this department. *B. pertussis* 2992 was from Wellcome Research Laboratories, Beckenham, Kent.

Plate cultures were grown on Cohen and Wheeler's (6) liquid medium containing, in addition, 5% (vol/vol) defibrinated horse blood and solidified with 1.5%

(wt/vol) agar (CWBA). Where required, NA or ND was added to a concentration of 500 μg/ml.

Shake-flask and semicontinuous cultures were grown in Stainer and Scholte's (32) defined liquid medium (SS), with NA and ND concentrations as recorded below.

Unless otherwise stated, all cultures were grown at 37°C.

For semicontinuous culture (27) of *B. pertussis*, 700 ml of culture was stirred continuously in a 1-liter glass fermentor which enabled the pH to be kept constant at 7.6 throughout growth and the temperature and oxygen tension to be monitored. At 24-h intervals, 600 ml of culture was drained off and replaced with 600 ml of fresh prewarmed medium, using the remaining 100 ml of culture as inoculum. Each culture medium exchange is referred to as a cycle.

NA and ND uptake. Uptake of NA and ND was measured by filtration, at timed intervals, of portions of assay mixture containing washed *B. pertussis* cells, phosphate buffer, glutamate, and either [^{14}C]NA or [^{14}C]ND (23).

Preparation of intact cells and culture supernatants for AC assays. Cultures on solid media were scraped into several volumes of 0.85% (wt/vol) sodium chloride. All cultures, both liquid and solid, were then harvested by centrifugation at 8,000 × *g* for 15 min at 4°C in a Sorvall Superspeed RC2-B centrifuge.

Culture supernatants were decanted, filter sterilized (25-mm diameter, 0.4-μm pore size cellulose acetate filters, Oxoid), and stored on ice until required.

Intact cells were washed once and suspended in either 0.85% (wt/vol) sodium chloride or 50 mM Tris-hydrochloride (pH 7.6) to a concentration of 0.5 to 5.0 mg (dry weight) per ml for assay in the presence of an ATP-regenerating system, or 20 to 30 mg (dry weight) per ml for assay in its absence.

Cell suspensions and culture supernatants were assayed immediately for AC activity.

Preparation of cell fractions for AC assay. Cell disintegrates of *B. pertussis* suspensions were prepared by rotary disintegration as described previously (27). The cell disintegrate was fractionated by differential centrifugation in a Sorvall RC2-B Superspeed centrifuge as follows: (i) cell debris, 3,000 × *g*, 15 min, 4°C; (ii) cell walls, 12,000 × *g*, 20 min, 4°C; (iii) outer membrane vesicles (OMV), 50,000 × *g*, 60 min, 4°C; (iv) soluble, the supernatant from the final 50,000 × *g* spin. All sediments were washed once, suspended in distilled water, and assayed immediately for AC activity.

Sucrose density gradient centrifugation. OMV preparations were layered on top of 15 to 70% (wt/vol) sucrose gradients in 30 mM Tricine (pH 7.8) and spun, in a Sorvall RC2-B fitted with a vertical rotor head, at 21,000 rpm for 30 min at 4°C and then at 15,000 rpm overnight at 4°C. Fractions (2 ml) were collected, and the optical density at 280 nm, specific gravity, and AC activities were measured.

AC assay. The assay for AC (EC 4.6.1.1) was adapted from that of Hewlett and Wolff (15). The reactions were done in a volume of 120 μl containing 60 mM Tricine (pH 8.0), 1 mM ATP, 10 mM $MgCl_2$, 0.1 to 5.0 mg (dry weight) of enzyme preparation per ml, and, where appropriate, an ATP-regenerating system of 6.7 mM creatine phosphate and 0.3 mg (dry weight) of creatine phosphokinase per ml. The reaction was stopped after 10 min of incubation at 30°C by the addition of 600 μl of solution containing 50 mM Tris-hydrochloride and 4 mM EDTA (pH 7.5); this was vortexed and heated for 5 min at 100°C.

The tubes were then spun for 20 min at 1,200 × *g* and 4°C in an MSE centrifuge with multitube carrier; 600 μl of each supernatant was removed and mixed with 150 μl of 500 mM Tris-hydrochloride–40 mM EDTA (pH 7.5), vortexed, and stored at −20°C. Each enzyme preparation was assayed in duplicate.

The product of the AC reaction, cAMP, was assayed by the cAMP binding protein determination of Gilman (12), with a cAMP Assay Kit purchased from Amersham.

Measurement of agglutinogen titers. Cell cultures were harvested as described for AC assays, except that they were resuspended to a concentration of 1.0 mg (dry weight) per ml in 0.85% (wt/vol) sodium chloride. Formalin was added to a final concentration of 0.25% (vol/vol) and the suspensions were incubated at 37°C for 24 h before storage at 4°C.

Agglutinogen factors 1, 2, and 3 were assayed by the method of Novotny and Cownley (27), with specific factor sera prepared according to Andersen (3). Serial two-fold dilutions of the sera were mixed with an equal volume (25 μl) of formolized cell suspension in microtiter hemagglutination trays. The trays were sealed, incubated at 56°C for 1 h, and left overnight at room temperature.

The agglutination titer recorded was the reciprocal of the highest dilution of serum giving agglutination of *B. pertussis*.

RESULTS

Effect of NA modulation on NA and ND uptake. The characteristics of NA and ND uptake by *B. pertussis* 134 were very similar when the cells were grown in medium NA 5, which contained 5 μg of NA per ml (23, 24). The effect of growth in medium containing 500 μg of NA (NA 500) or of ND (ND 500) per ml on NA and ND uptake by *B. pertussis* 134 is illustrated in Table 1. The NA uptake rate was reduced by 66%, and the ND uptake rate was reduced 74% in cells harvested from NA 500 growth medium. By contrast, the rate of NA uptake of cells grown in ND 500

TABLE 1. Effect of different NA and ND concentrations in the growth medium on the uptake of ^{14}C-labeled NA and ND by *B. pertussis* 134

Growth medium[a]	Rate of uptake (pmol/min per mg [dry wt] ± SEM) of:		Envelope polypeptides[b]
	NA	ND	
NA 5	9.0 ± 1.2	8.5 ± 1.1	+
NA 500	3.1 ± 0.1	2.2 ± 0.4	−
ND 500	7.1 ± 1.6	4.7 ± 1.3	+

[a] Numbers refer to the concentrations (in micrograms per milliliter) of NA or ND in the liquid growth medium.

[b] Presence (+) or absence (−) of the 28K and 30K envelope polypeptides (34).

TABLE 2. Intact-cell AC activity of *B. pertussis* 134 and 18323 grown on CWBA alone and with additional NA 500 or ND 500

Strain	Substance added to CWBA medium (μg/ml)[a]	Intact-cell AC activity[b] (pmol of cAMP per min/mg [dry wt])
134	None	700
	NA 500	0
	ND 500	720
18323	None	20
	NA 500	0
	ND 500	330

[a] Cultures were grown for 48 h at 37°C, subcultured on the same medium, and assayed for AC activity without an ATP-regenerating system.
[b] Measured in the absence of an ATP-regenerating system.

medium was reduced by only 21%, whereas ND uptake was reduced by 45%.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of cell envelopes from these cultures showed that the 28,000- and 30,000-dalton (28K and 30K) polypeptide bands were lost only after growth in NA500 (34).

Effect of NA modulation on AC. The effect of growth in high-NA and high-ND media on AC activity was studied in defined solid media, in shake-flask cultures, and in semicontinuous culture. This was done with several strains and in the presence and absence of an ATP-regenerating system.

In the experiments with growth on solid media, strains 134 and 18323 were incubated at 37°C on CWBA with additional NA or ND at 500 μg/ml. AC activity was measured with intact cells in the absence of an ATP-regenerating system (Table 2). Intact-cell AC activity in *B. pertussis* 18323 was considerably lower than that of strain 134 when the cultures were grown on CWBA. When NA 500 was added to the medium, no intact-cell AC activity was detectable in either strain. Addition of ND 500 to the medium had no effect on the AC activity of strain 134, but the activity of strain 18323 was considerably enhanced.

Attention was next given to cultures in liquid media. *B. pertussis* 134 and 2992 were grown for two subcultures at 37°C in shake flasks with NA 5, NA 500, or ND 500. AC activities of intact cells, cell disintegrates, and culture supernatants were assayed in the presence of an ATP-regenerating system (Table 3). As with growth on solid medium, *B. pertussis* cells harvested from liquid medium with high NA exhibited greatly reduced AC activity, not only in intact cells but also in cell disintegrates and culture supernatants. Growth in the corresponding medium containing high ND had little effect on the AC activities.

Modulation of *B. pertussis* by NA 500, as measured by the marked reduction of AC activity, required more than one subculture in the medium with high NA (Table 3). At the first subculture, AC activity in both strains was more reduced in the culture supernatant than in the intact cells. After the second subculture, the AC activity of the intact cells was much reduced, and the activity in the culture supernatant was lowered still further.

Experiments with semicontinuous culture (SCC) were done by using *B. pertussis* 2992. SCC differs from sequential subcultures in shake flasks in that the pH of the medium is kept constant at 7.6 throughout growth. In shake-

TABLE 3. Effect of NA and ND concentrations and of subculture in shake flasks on the AC activity of *B. pertussis* 134 and 2992

Strain	Substance added to SS medium (μg/ml)	Subculture no.	AC activity[a]		
			Intact cells[b]	Cell disintegrate[b]	Culture supernatant[c]
134	NA 5	1	12.2	NT[d]	87.4
		2	13.4	47.4	46.5
	NA 500	1	7.7	NT	7.4
		2	0.1	0.2	0.05
	ND 500	2	11.8	53.0	17.3
2992	NA 5	1	27.3	NT	89.3
		2	7.6	62.8	48.8
	NA 500	1	2.9	NT	7.1
		2	0.02	0.5	0.2
	ND 500	2	17.6	52.7	44.3

[a] Measured in the presence of an ATP-regenerating system.
[b] Expressed as nanomoles of cAMP formed per minute per milligram (dry weight).
[c] Expressed as nanomoles of cAMP formed per minute per milliliter.
[d] NT, Not tested.

TABLE 4. Effect of growth conditions in SCC on the AC activity of *B. pertussis* 2992[a]

Sequential growth conditions		No. of cycles	AC activity in:		
NA concn (μg/ml)	Temp (°C)		Intact cells[b]	Cell disintegrate[b]	Culture supernatant[c]
5	37	10	4.6	22.8	NT[d]
500	37	5	5.7	12.4	0.05
5	37	6	11.5	48.4	0.2
5	28	6	0.6	0.9	0

[a] Cultures were grown under the appropriate conditions for the stated number of cycles before AC assays.
[b] Expressed as nanomoles per minute per milligram (dry weight).
[c] Expressed as nanomoles per minute per milliliter.
[d] NT, Not tested.

flask culture, the pH rose towards the onset of stationary phase, as previously reported (30), and growth stopped when the pH reached 8.2 to 8.3. Cells from SCC were assayed for AC activity in the presence of an ATP-regenerating system after 5 to 10 subcultures in each medium. The sequential changes in growth conditions were: NA 5, 37°C; NA 500, 37°C; NA 5, 37°C, and NA 5, 28°C. The latter growth condition yields antigenically modulated cells (21) and is known to cause the loss of AC activity (28).

When grown with NA 500 in SCC, *B. pertussis* 2992 did not lose AC activity (Table 4), although AC activity was markedly reduced when growth was at 28°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of cell envelopes from these cultures showed that the 28K and 30K bands were lost only when growth was at 28°C. Thus, the presence or absence of these envelope polypeptides and of AC activity were correlated.

AC activity in the outer membrane of *B. pertussis*. Hewlett et al. (14) and Cowell et al. (8) reported that about 90% of the AC activity measurable in intact cells was extracytoplasmic, and that a large proportion of it was sited in the periplasm but loosely associated with the cell envelope. The description of a method for preparing OMV of *B. pertussis* (27) enabled us to measure the amount of intact-cell AC activity associated with the outer membrane and the effect of NA modulation on cellular distribution. In nonmodulated shake-flask cultures, between 3 and 6% of the intact cell AC activity was associated with the OMV when measured in the presence of an ATP-regenerating system (Table 5). In NA-modulated cells, the AC activity associated with the OMV was reduced, as was the AC activity in the cell wall fraction. This latter fraction contained adhering outer membrane and cytoplasmic membrane remnants (27).

The AC of intact cells of *B. pertussis* 2992 was unaffected by high NA during growth in SCC (Table 4). The percentage of AC activity in the OMV from these cultures remained constant at about 10% when measured in the presence of an ATP-regenerating system. The OMV preparations from the cultures listed in Table 4 were further purified on sucrose density gradients. AC activity was found only in those gradient fractions containing OMV. The percentage of AC activity in the OMV fractions quoted here should be maximal because the ATP-regenerating system contained a contaminant, possibly calmodulin (5, 13, 16, 35), which stimulated the activity of membrane-associated AC to a greater

TABLE 5. Distribution of AC activity in cell fractions of *B. pertussis* grown in shake flasks; effects of NA modulation

Strain	Substance added to SS medium (μg/ml)	% AC activity[a] in:			
		Cell debris	Cell walls	OMV	Soluble
134	NA 5	31	10	2	56
	NA 500	34	6	2	58
	ND 500	36	13	6	45
2992	NA 5	39	14	5	42
	NA 500	38	5	1	56
	ND 500	32	16	6	46

[a] AC activity was measured in the presence of an ATP-regenerating system at the second subculture in each medium.

TABLE 6. Effect of NA modulation in shake flask cultures in SS medium on the agglutinogens of *B. pertussis* 134 and 2992

Strain	Substance added to SS medium (μg/ml)	Reciprocal agglutinogen titer of factor:		
		1	2	3
134	NA 5	32	256	32
	NA 500	512	0	0
	ND 500	32	32	32
2992	NA 5	64	128	64
	NA 500	1,024	0	0
	ND 500	128	128	32

extent than soluble AC. The AC activity in the OMV preparations of the cultures listed in Table 4 was only about 2% when assayed in the absence of an ATP-regenerating system.

Effect of NA modulation on the heat-labile agglutinogens. The titers of agglutinogens 1, 2, and 3 of *B. pertussis* 134 and 2992 grown for two subcultures in shake flasks were measured (Table 6). Growth in ND 500 had no effect on the titers compared with growth in NA 5. With both strains, agglutinogens 2 and 3 were lost during growth in NA 500; the titer of factor 1, however, was markedly increased.

The titers of these agglutinogens in strain 2992 grown in SCC were also measured (Table 7). Growth with NA 500 at 37°C had no effect on the titers of the three agglutinogens, but when growth was at 28°C, the agglutinogens 2 and 3 were lost. However, agglutinogen 1 was unaffected. In these latter cultures, agglutinogen 3 was lost after four cycles and agglutinogen 2 was lost only after five cycles, suggesting a sequential order of loss and a requirement for cell division.

DISCUSSION

Growth in high concentrations of ND has been shown to repress the uptake of ND by *Streptococcus faecalis* (26). Likewise, a cytoplasmic membrane-located NA-binding protein in *Lactobacillus casei* was repressed by growth in high NA concentrations. The reduction in uptake of NA and ND by *B. pertussis* grown in high concentrations of those vitamins is, therefore, not unexpected. However, as in many other bacteria (11), ND is rapidly metabolized to NA in *B. pertussis* (W. L. McPheat, unpublished data) by the enzyme ND deamindase. The presence of this enzyme in *B. pertussis* suggests a common metabolism of the two vitamins via the ubiquitous pyridine nucleotide cycle (11).

Growth in high concentrations of both NA and ND is known to cause repression of at least one of the enzymes of the pyridine nucleotide cycle in *Escherichia coli* and *Salmonella typhimurium* (4, 10). Such repression would also contribute to a reduction in vitamin uptake. In *B. pertussis*, however, growth in ND 500 had only a modest effect on ND uptake and no effect at all on NA uptake. By contrast, growth in NA 500 had a marked and similar inhibitory effect on the uptake of both vitamins.

These results, in conjunction with the effect of structural analogs on NA and ND uptake by *B. pertussis* (24), suggest that NA and ND have separate transport systems into the *B. pertussis* cell, followed by a common metabolism via the pyridine nucleotide cycle.

In shake-flask cultures or cultures on solid, blood-containing media, growth with NA 500, but not with ND 500, resulted in a marked reduction of AC levels in *B. pertussis* and of agglutinogen factors 2 and 3 but not factor 1. The reduction in AC activity in intact cells was due neither to excess excretion of AC into the culture supernatant, nor to a decreased permeability of the cell envelope (as cell disintegrate AC activity was also much reduced), nor to a gross rearrangement of the localization of AC in intact cells. The reduction of AC activity and agglutinogen titers continued for at least two subcultures (approximately six to eight generations). This is similar to the finding by Lacey (21) that antigenic modulation required approximately seven cell generations to become established.

Modulation by NA appears, however, to be dependent on both the strain and the type of growth conditions. Strain 18323 showed a loss of AC activity with NA 500 growth but a marked increase in AC activity when grown with ND 500. This strain was reported by Adams (2) not to undergo antigenic modulation and by Pusztai and Jóo (29) not to lose agglutinability after NA 500 growth. This strain, therefore, may well be atypical of *B. pertussis* strains, and its high intracerebral virulence in mice may be linked to this metabolic peculiarity.

Growth of strain 2992 under conditions of constant pH (semicontinuous culture) permitted the induction of antigenic modulation but not of NA modulation (Tables 4 and 7). This lends further support to the suggestion by Pusztai and

TABLE 7. Effect of growth conditions in SCC on the agglutinogens of *B. pertussis* 2992

NA concn (μg/ml)	Temp (°C)	SCC cycle no.	Reciprocal agglutinogen titer of factor:		
			1	2	3
5	37	8	128	128	32
500	37	4	128	128	32
5	37	5	512	512	32
5	28	5	128	0	0

Jóo (29) that these two types of modulation are functionally distinct.

The presence of ND deamidase activity in *B. pertussis*, converting ND to NA (McPheat, unpublished data), suggests that NA and ND must interact with separate cell surface components in order for ND 500 growth not to give rise to NA modulation. The nature of the regulatory component with which NA may interact is unknown.

In a recent article, Schneider and Parker (31) reported that 6-chloronicotinic acid and quinaldic acid were more effective modulating agents than NA. These authors also identified 2-chloronicotinic acid and isoniazid as substances that interfered with NA modulation. Effects on AC and individual agglutinogens were not reported but would clearly be of interest.

Confer and Eaton (7) reported that cell-associated AC may be of importance to the virulence of *B. pertussis*. We have shown in this report that a small proportion of the AC is associated with the outer membrane of *B. pertussis*. The response of the AC and other outer membrane components, such as the agglutinogens, to NA and ND concentrations may be of importance in understanding of *B. pertussis* pathogenicity.

ACKNOWLEDGMENTS

This investigation was supported by grants from the Science and Engineering Research Council and from Wellcome Research Laboratories.

We thank Kevin Cownley for his technical assistance.

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