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Conversion of *Bordetella pertussis* to *Bordetella parapertussis*

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SUMMARY

The epidemiological and drug susceptibility data on whooping cough suggested a possibility that *Bordetella pertussis* converts in some way to *Bordetella parapertussis*. To prove this, *B. pertussis* strain 75 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and a mutant resistant to staphcillin V and eight mutants resistant to trimethoprim were isolated. The staphcillin V-resistant mutant of *B. pertussis* agreed with all of the criteria of *B. parapertussis* and the trimethoprim-resistant mutants also agreed with many of these criteria. Thus, a hypothesis is presented that *B. parapertussis* is a mutant of *B. pertussis* which appeared in nature probably by a selective pressure of antibiotics.

INTRODUCTION

Since 1955, widespread vaccination against whooping cough has been carried out by the use of *Bordetella pertussis* strain Tohama phase I, and the morbidity and the mortality have been drastically reduced in Japan (Miyamoto *et al.* 1974). During a period from June 1972 to February 1974, there was a rare outbreak of whooping cough in Miura Peninsula district (Miyamoto *et al.* 1974). From cases in the epidemic, 71 strains of *B. pertussis* and 8 strains of *B. parapertussis* were isolated (Sekiya *et al.* 1975). In view of the fact that a rare epidemic of pertussis was immediately followed by one of parapertussis, a pathogenic relatedness of these two micro-organisms was suspected.

In Denmark (Lautrop, 1971), *B. pertussis* and *B. parapertussis* infections presented epidemic waves at 4 year intervals with a 2 year shift between the pertussis and the parapertussis peaks. After introduction of vaccination against pertussis, both isolates were reduced simultaneously. These data also suggested the same possible pathogenic relationship of the two micro-organisms.

According to the data of drug susceptibility patterns of the two species (Watanabe *et al.* 1975), there are two types of antibiotic or chemotherapeutic substances; one to which *B. pertussis* strains are more susceptible than *B. parapertussis* strains, and the other to which the two organisms are equally susceptible. It is characteristic that there is no drug to which *B. pertussis* strains are less susceptible than *B. parapertussis* strains. This characteristic drug resistance pattern is also observed in many authentic strains of these two species.

Based on these data, especially those of drug resistance pattern, we initially

suspected that *B. parapertussis* might be converted upon transfer of a kind of R plasmid to *B. pertussis*. To confirm this, efforts were made to isolate a trimethoprim (TMP)-resistant mutant of *B. pertussis*. As TMP is active to the same degree to these two species, we expected to isolate *B. parapertussis* resistant to TMP when the TMP-resistant mutant of *B. pertussis* was mixed with an authentic strain of *B. parapertussis* sensitive to TMP. However, all of eight TMP-resistant mutants of *B. pertussis* thus obtained were shown to be very similar to *B. parapertussis*. This observation suggested that conversion of *B. pertussis* may be through pleiotropic mutation. Efforts were turned to isolate a staphcillin V (SP)-resistant mutant of *B. pertussis* because *B. parapertussis* is less susceptible to SP than *B. pertussis*.

In this paper we describe the results obtained with these mutants and discuss the possibility that *B. parapertussis* is a pleiotropic mutant of *B. pertussis*.

MATERIALS AND METHODS

Bacterial strains

B. pertussis strains, 75 (phase I), Tohama (phase I), and Sakairi (phase III), and *B. parapertussis* strains, 77 and 79, were kindly provided by Dr K. Sekiya, Kitasato Institute, Tokyo. A *B. parapertussis* strain, 17903, was kindly given by Dr Y. Sato, National Institute of Health, Tokyo.

Cultures

The freeze-dried cultures were grown for 72 h and subcultured on Bordet–Gengou (BG) agar plates for 48 h at 37 °C. Nutrient agar plates supplemented with 0·1 % tyrosine were used for growth pigmentation tests.

Isolation of staphcillin V-resistant mutants

Cells grown on BG agar were spread again on a fresh BG agar plate. Microcrystalline *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was streaked on the agar surface. After 48 h incubation at 37 °C, the cells grown on the BG agar with NTG were grown again on drug-free BG agar and then selected on BG agar supplemented with 200 μg. staphcillin V per ml. The colonies formed were purified and used as staphcillin V (SP)-resistant mutants.

Isolation of trimethoprim-resistant mutants

Cells grown on BG agar were spread again on a fresh BG agar plate. Microcrystalline NTG and 5 mg of trimethoprim (TMP) dissolved in 0·05 ml of dimethylsulphoxide were cross-streaked on the agar surface. After incubation for 6 days at 37 °C, the cells grown were harvested and respread on BG agar supplemented with 500 μg. TMP per ml. The colonies formed were purified and used as TMP-resistant mutants.

Drug susceptibility tests

The replica-plating method was adopted using BG agar plates containing serial dilutions of antibiotics. The minimal growth inhibitory concentration (MIC) of each antibiotic was determined after 48 h incubation at 37 °C.

Determination of the buoyant density of deoxyribonucleic acid

Deoxyribonucleic acid (DNA) was obtained using the procedure of Marmur (1961). The buoyant density of DNA was determined using the procedure of Schildkraut, Marmur & Doty (1962).

Biochemical characterization

Urea splitting was examined in Urease test broth (Eiken) after incubation at 37 °C for 24 h. Citrate utilization was examined on a Christensen citrate agar slant (Eiken) after incubation at 37 °C for 7 days.

Preparation of disrupted cell supernatant

Cells were suspended in 0·05 M phosphate buffer (pH 8·0) containing 0·5 M-NaCl, disrupted in an Insonator Model 200 M (Kubota Co., Tokyo) ultrasonic-oscillator for 10 min at 200 W full power and centrifuged at 20000 ***g*** for 20 min. The protein content of the supernatant was assayed by the method of Lowry *et al.* (1951).

Haemagglutination

Various dilutions of the disrupted cell supernatant in 0·5 ml volumes were mixed with 0·025 ml of 2% (v/v) horse erythrocyte suspension and incubated for 1 h at 37 °C followed by storage overnight at 4 °C.

Animal tests

The dermonecrotic effect was tested in guinea pigs by injecting 0·1 ml of various dilutions of the disrupted cell supernatant intracutaneously. The necrotic lesions were observed at 24 h and the minimal amount of protein required to give a necrotic lesion larger than 10 mm in diameter was determined. For the histamine sensitizing test, the method of Niwa (1962) was used.

Agglutination

Overnight cultures on BG agar plates were suspended, centrifuged and re-suspended in saline at a concentration of 18 mg wet weight per ml. One loopful of the saline suspension was mixed with one loopful of serum dilutions on a slide. The results were read within 1 min.

Antisera

Anti-pertussis K, anti-pertussis agglutinogen 1 and anti-pertussis O sera were obtained from Dr Y. Sato, National Institute of Health, Tokyo. Anti-parapertussis K serum was obtained from Dr K. Sekiya, Kitasato Institute, Tokyo.

Anti-77 serum was rabbit antiserum immunized with *B. parapertussis* strain 77 absorbed with *B. pertussis* strain 75.

Chemicals

Trimethoprim lactate (TMP) and nalidixic acid were kindly given by the Japan Branch of the Wellcome Foundation and by Daiichi Pharmaceutical Co., Tokyo, respectively. Other antibiotics were commercially purchased.

RESULTS

Isolations of drug resistant mutants

Overnight cultures of *B. pertussis* strain 75 were treated with NTG and an SP-resistant strain, NK 1 and eight TMP-resistant strains, NK 2, NK 3, NK 4, NK 5, NK 6, NK 7, NK 8 and NK 9, were obtained.

DNA densities of the mutants were $1{\cdot}726 \pm 0{\cdot}001$ g ml^{-1} which were in good agreement with those of *B. pertussis* and *B. parapertussis* strains within the experimental error (Bacon *et al.* 1967).

Drug susceptibility tests

MIC values of drugs for the mutants as well as for the parental and authentic strains are shown in Table 1. These data are in good agreement with those of Watanabe *et al.* (1975). *B. pertussis* phase I strains were far more susceptible to nalidixic acid, SP, streptomycin, penicillin G and ampicillin than *B. parapertussis* strains, but their susceptibility to tetracycline, chloramphenicol and TMP was similar to that of the *B. parapertussis* strains. The susceptibility range of *B. pertussis* strain Sakairi (phase III) was shifted slightly towards that of the *B. parapertussis* strains, but the range for strain NK1 was very different from the ranges of *B. pertussis* strains, and similar to those of *B. parapertussis* strains. The susceptibility ranges of eight strains resistant to TMP were shifted in varying degrees towards those of *B. parapertussis*.

Cultural, morphological and biological characters of the mutants

On BG agar plates, mutants other than strains NK 6 and NK 7 formed large, dark greenish, smooth colonies, 1–2 mm in diameter, with a haemolytic zone within 48 h (Table 2). In confluent growth, mutants produced black pigmentation on BG agar plates. The mutants also grew on nutrient agar plates supplemented with tyrosine with brown pigmentation of the medium. The colony sizes of strains NK 6 and NK 7 on BG agar plates were small (0·4–0·5 mm in diameter) but other characters of the strains were the same as those of other TMP-resistant mutants. These characters are all in agreement with those of *B. parapertussis* strains but not with those of *B. pertussis* strains (Table 2).

Mutants were all Gram-negative coccoid forms with a distinct capsule-like sheath. Occasionally long chains of cells with a common sheath were observed. Cell chains were clear in capsule stain preparations. In Gram-stain preparations, rod-like shapes rather than cell chains were observed.

Table 1. *Minimal growth inhibitory concentrations of antibiotics for the mutants*

Strain	Minimal growth inhibitory concentration (μg/ml)							
	TC	CM	NAL	SP	SM	PC	ABPC	TMP
B. pertussis								
75	0·8	0·8	3·1	6·25	1·6	1·0	0·8	6·25
Tohama	1·6	0·8	3·1	12·5	1·6	1·0	0·8	6·25
Sakairi	1·6	1·6	6·25	≧ 6400	6·25	16	3·1	12·5
B. parapertussis								
77	3·1	1·6	25	≧ 6400	200	≧ 500	200	12·5
79	3·1	3·1	100	≧ 6400	50	≧ 500	400	12·5
Mutant								
NK 1	3·1	3·1	100	≧ 6400	200	≧ 500	200	12·5
NK 2	0·8	1·6	400	100	25	64	50	1600
NK 3	0·8	12·5	200	50	25	64	50	1600
NK 4	1·6	12·5	400	400	25	64	25	1600
NK 5	3·1	3·1	400	400	6.25	64	25	≧ 3200
NK 6	0·8	12·5	200	50	1·6	8	12·5	1600
NK 7	1·6	1·6	200	25	1·6	16	6·25	≧ 3200
NK 8	1·6	1·6	200	50	1·6	32	50	1600
NK 9	1·6	12·5	200	400	1·6	32	50	≧ 3200

TC, tetracycline; CM, chloramphenicol; NAL, nalidixic acid; SP, staphcillin V; SM, streptomycin; PC, penicillin G; ABPC, aminobenzylpenicillin; TMP, trimethoprim.

Table 2. *Cultural, morphological and biochemical characters of the mutants*

	B. pertussis		TMP-resistant mutant	SP-resistant mutant	*B. parapertussis*
	Phase I	Phase III			
Colonies on BG agar					
Diameter (mm)	0·2–0·5	0·2–0·5	0·4–2·0	1·0–2·0	1·0–2·0
Colour	Greyish white	Greyish white	Dark green	Dark green	Dark green
Pigmentation of the agar	−	−	+	+	+
Nutrient agar supplemented with tyrosine					
Growth	−	+	+	+	+
Pigmentation of the agar	−	−	+	+	+
Chain formation of cells	−	−	+	+	+
Urea splitting	−	−	−	+	+
Citrate utilization	−	−	± *	+	+
Sensitize mice to histamine	+	NT**	−	−	−

*, Slightly utilized; **, not tested.

Urea was split by the SP-resistant mutant but not by the TMP-resistant mutants. Citrate was utilized by the SP-resistant mutant. Slight or no citrate utilization was observed in the TMP-resistant mutants (Table 2).

All mice injected with the heated cell suspensions of *B. pertussis* strains intravenously were dead within 30 min after intraperitoneal injection of histamine.

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Table 3. *Haemagglutinating and dermonecrotic activities of the mutants*

Strain	Minimal haemagglutinating dose (μg protein/ml)	Minimal dose to produce dermonecrotic lesion of 10 mm in diameter (μg protein)
B. pertussis		
75	0·8	1·25
Tohama	1·6	0·625
B. parapertussis		
77	1·6	2·5
17903	400	2·5
Mutant		
NK 1	3·1	2·5
NK 2	400	10

Table 4. *Agglutination tests with mutants*

	Antiserum						
	Anti-pertussis			Anti-parapertussis		Anti-mutant	
Antigen	Anti-K	Anti-agglutinogen 1	Anti-O	Anti-77	Anti-K	Anti-NK 1	Anti-NK 2
Pertussis							
Tohama	320	4	80	< 10	< 10	40	< 10
75	80	2	320	< 10	< 10	< 10	< 10
Sakairi	< 10	< 1	80	< 10	< 10	< 10	< 10
Parapertussis							
17903	< 10	< 1	40	2560	160	2560	< 10
77	< 10	< 1	160	640	160	640	< 10
Mutant							
NK 1	< 10	< 1	40	640	80	2560	< 10
NK 2	< 10	< 1	20	< 10	< 10	< 10	160

No death of mice was observed with any of the mutants or with *B. parapertussis* (Table 2).

Haemagglutinating and dermonecrotic activities were observed using the disrupted cell supernatants (Table 3). *B. pertussis* strains and *B. parapertussis* strain 77 showed considerable haemagglutinating activity, but strain 17903 did not. Mutant NK 1 also showed haemagglutinating activity, but not NK 2. Dermonecrotic activity was high with *B. pertussis* strains but low with *B. parapertussis* strains. Mutant strain NK 1 had a dermonecrotic activity similar to that of the *B. parapertussis* strains but the activity of NK 2 was much lower.

Agglutination tests with mutants

The results of agglutination tests are shown in Table 4. Cells of *B. pertussis* phase I strains reacted with anti-pertussis specific sera and an anti-O serum but

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not with anti-parapertussis specific sera. Cells of *B. pertussis* strain Sakairi (phase III) reacted only with an anti-O serum. Cells of *B. parapertussis* strains reacted with anti-parapertussis specific sera and an anti-O serum but not with anti-pertussis specific sera. Cells of mutant NK 1 (SP-resistant mutant) reacted with anti-parapertussis specific sera and anti-O serum but not with anti-pertussis specific sera. Cells of strain NK 2 reacted with an anti-O serum but not with anti-pertussis and anti-parapertussis specific sera. Cells of other TMP resistant mutants showed similar results to those of strain NK 2 (data not shown). The antiserum to strain NK 1 reacted with cells of strain NK 1 and *B. parapertussis* strains. A weak reaction of anti-NK 1 serum with cells of *B. pertussis* strain Tohama might be attributed to the presence of anti-O antibodies. The antiserum to strain NK 2 reacted only with cells of strain NK 2 and of other TMP-resistant mutants but not with cells of any other strains. C-mode cells of *B. pertussis* strain 75, which were obtained on EM $MgSO_4$ 4·0 agar (Lacey, 1960) did not react with any antisera which we used. These results suggest that strain NK 2 does not consist of C-mode cells.

DISCUSSION

According to *Bergey's Manual of Determinative Bacteriology*, 8th ed. (1974), *B. pertussis* differs from *B. parapertussis* in many characters. In *B. pertussis* there is phase variation from the typical phase I via the intermediate to the phase III (Nakase, Takatsu & Kasuga, 1969), and many characters of phase III are similar to those of *B. parapertussis*. *B. parapertussis* is distinguished therefore from *B. pertussis* by only few characters; that is, browning of peptone agar or nutrient agar supplemented with tyrosine at 0·1% or black pigmentation of BG agar, urea splitting, citrate utilization, loss of histamine sensitivity (*Bergey's Manual of Determinative Bacteriology*, 8th ed., 1974), drug susceptibility pattern (Watanabe *et al.* 1975), and specific K antigens.

All the mutants showed browning and black pigmentation. Urea was split by the SP-resistant mutant but not by the TMP-resistant mutants. Citrate was also utilized in various degrees by these mutants. Loss of histamine sensitizing activity was also observed in these mutants. *B. pertussis* phase I strains were far more susceptible to some drugs than *B. parapertussis* strains. The susceptibility pattern of *B. pertussis* strain Sakairi (phase III) was intermediate between those of *B. pertussis* phase I and of *B. parapertussis*. The susceptibility pattern of the SP-resistant mutant was similar to that of *B. parapertussis*. The susceptibility patterns of eight TMP-resistant mutants were shifted in various degrees towards those of *B. parapertussis*. Agglutination data were of particular interest because anti-parapertussis specific sera reacted with the cells of the SP-resistant mutant obtained by selecting with a drug selective for *B. parapertussis* but not with the cells of the TMP-resistant mutants obtained by selecting with a drug not selective for *B. parapertussis*. However, these serological data should not be overestimated because the antigenic structure of *B. pertussis* is easily converted by subculturing (Stanbridge & Preston, 1974) or by change of environmental conditions of growth (Lacey, 1960). Strain NK 2 had no haemagglutinating or dermonecrotic activity

which may reflect an alteration of the cell surface structure. An antiserum to the strain NK 2 did not react with C-mode cells suggesting the existence of antigenic variations other than the already known phase variation (Nakase *et al.* 1969) or the antigenic modulation (Lacey, 1960).

Nothing is known about the biochemical basis underlying this pleiotropic mutation, but none of the changes observed are contradictory to a supposition that the mutation has altered a permeability mechanism of the cell membrane.

Isolation of a revertant with the same characters as the original *B. pertussis* is desirable but has not yet been observed because all the characters *in vitro* are selectively advantageous for the *parapertussis*-type mutants.

Thus, the conclusions that the SP-resistant mutant is in fact *B. parapertussis* and that the eight TMP-resistant mutants and the strain Sakairi (phase III) are intermediates between *B. pertussis* and *B. parapertussis* appear to us to have been substantiated.

These conclusions are also in good agreement with the epidemiological data on whooping cough epidemics in Miura Peninsula (Miyamoto *et al.* 1974) and in Denmark (Lautrop, 1971). For example, Lautrop (1971) reported that epidemic waves of pertussis and parapertussis occurred at 4-year intervals in Denmark and that since the introduction of pertussis vaccination the size of epidemics of the two diseases had been reduced gradually and almost simultaneously. He considered a direct influence of pertussis vaccination on the reduction in the incidence of parapertussis as unlikely because cross-immunity in the normal sense of words does not exist between these two infections. In view of our data presented here, however, the reduction of parapertussis cases may result not from the cross-immunity but from the reduction of pertussis cases itself as a consequence of pertussis vaccination. A single epidemic of parapertussis without the preceding epidemic of pertussis may not exist or may at most be very rare. If it is true, parapertussis should also be controlled by the pertussis vaccination.

Finally we should like to point out a possibility that *B. parapertussis* is more easily selected in an epidemic of pertussis than the original pathogen, *B. pertussis*, because the former is always less susceptible to many conventional antibiotics which is selectively advantageous. This may have added another problem to the risk caused by the misuse of antibiotics.

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REFERENCES

Bacon, M. F., Overend, W. G., Lloyd, P. H. & Peacocke, A. R. (1967). The isolation, composition and physicochemical properties of deoxyribonucleic acid from *Bordetella pertussis*. *Archives of Biochemistry and Biophysics* **118**, 352–61.

Buchanan, R. E. & Gibbons, N. E. (eds.) (1974). *Bergey's Manual of Determinative Bacteriology*, 8th ed. Baltimore: Williams & Wilkins.

Lacey, B. W. (1960). Antigenic modulation of *Bordetella pertussis*. *Journal of Hygiene* **58**, 57–93.

Lautrop, H. (1971). Epidemics of parapertussis. *Lancet* i, 1195–7.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin reagent. *The Journal of Biological Chemistry* **21**, 363–8.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *Journal of Molecular Biology* **3**, 208–18.

Miyamoto, Y., Takizawa, K., Sanetaka, T., Takahashi, T., Takamiya, A., Yamada, M., Nakase, Y., Watanabe, T., Hirose, M., Niwa, T., Fujiki, Y. & Aoki, Y. (1974). An epidemic survey and clinical examination of a regional outbreak of whooping cough. *The Journal of the Japanese Association for Infectious Diseases* **48**, 179–91. (In Japanese.)

Nakase, Y., Takatsu, K. & Kasuga, T. (1969). Antigenic structure and phase variation in *Bordetella pertussis*. *Japanese Journal of Microbiology* **13**, 283–91.

Niwa, M. (1962). Histamine sensitizing factor of *Bordetella pertussis*. *The Journal of Biochemistry* **51**, 222–91.

Schildkraut, C. L., Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. *Journal of Molecular Biology* **4**, 430–43.

Sekiya, K., Watanabe, M., Yamada, M. & Nakase, Y. (1975). Serotyping of recently isolated strains of *Bordetella pertussis*. *Japanese Journal of Bacteriology* **30**, 507–13. (In Japanese.)

Stanbridge, T. N. & Preston, N. W. (1974). Variation of serotype in strains of *Bordetella pertussis*. *Journal of Hygiene* **73**, 305–10.

Watanabe, M., Sekiya, K., Nakase, Y. & Yamada, M. (1975). Drug susceptibility of *Bordetella pertussis* and *Bordetella parapertussis*. *Japanese Journal of Bacteriology* **30**, 501–5. (In Japanese.)