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206

CHARACTERIZATION OF THE NONFERMENTATIVE GRAM NEGATIVE RODS OF GROUP IIK UTILIZING BIOCHEMICAL, SEROLOGICAL, AND ANTIBIOTIC SUSCEPTIBILITY TESTS

by

Donna Granstrom

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfullment of the Requirements for the Degree of Master of Science

May

1978

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VITA

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LIST OF TABLES

Table		Page
1.	Biochemical Reactions of 41 Strains of Group IIk Utilizing the Conventional Weaver Method of Identification	31
2.	Summary of the Reactions of 17 Strains of Nonfermentative Rod IIk-1 When Tested by Weaver's Scheme	35
3.	Summary of the Reactions of 24 Strains of Nonfermentative Rod IIk-2 When Tested by Weaver's Scheme	36
4.	Biochemical Reactions of ATCC Control Cultures Using Weaver's Identification Method	37
5.	Biochemical Reactions of 41 Strains of Group IIk Utilizing the API System of Identification	39
6.	Summary of the Reactions of 17 Strains of Nonfermentative Rod IIk-1 When Tested by the API 20 E System	43
7.	Summary of the Reactions of 24 Strains of Nonfermentative Rod IIk-2 When Tested by the API 20 E System	44
8.	Biochemical Reactions of ATCC Control Cultures Using the API Identification System	46
9.	Kirby-Bauer Susceptibility Test Results for 41 Strains of Group IIk	47
10.	Kirby-Bauer Susceptibility Test Results for ATCC Control Organisms	51
11.	Antimicrobial Susceptibility Patterns of Groups IIk-1 and IIk-2	52

Table | Page

12. Agglutination Reactions of 14 Anti-IIk-1 Antisera and Cross-Reacting Organisms. 55

13. Agglutination Reactions of 14 Anti-IIk-2 Antisera and Cross-Reacting Organisms. 57

14. Agglutination Reactions of 5 Anti-ATCC Control Antisera and Cross-Reacting Organisms. 60

15. Indirect Fluorescent Antibody Stain Results on 13 Strains of Group IIk-2. 62

16. Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-76-934 65

17. Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-LMC 4. 66

18. Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-K 2. 67

19. Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-LMC 1. 68

20. Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-C 1196 69

21. Agglutination Reactions of IIk and Control Strains with Unadsorbed and Adsorbed Anti-1852G 70

22. Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-1854G 71

23. Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-2232G 72

Table | Page

24. Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-74-1543 73

25. Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-2147G 74

26. Agglutination Reactions of IIk and Control Strains with Unadsorbed and Adsorbed Anti-C 1822 75

27. Agglutination Reactions of IIk and Control Strains with Unadsorbed and Adsorbed Anti-C 198 76

28. Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-2146G 77

29. Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-76-946 . . . 78

30. Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-C 499 . . . 79

31. Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-B 9042 . . . 80

32. Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-B 8684 . . . 81

LIST OF ABBREVIATIONS FOR BIOCHEMICAL STUDIES

A = Acid

BHIA = Brain heart infusion agar

DNAse = Deoxyribonuclease

F/N = Fluorescence-denitrification

g = Green

K = Alkaline

ly = Light yellow

ONPG = O-nitrophenyl-β-D-galactopyranoside

PBHB = Poly-β-hyroxybutyrate

SS = Salmonella-Shigella

TDA = Tryptophan deaminase

TSA = Trypticase soy agar

TSB = Trypticase soy broth

TSI = Triple sugar iron agar

V-P = Vogues-Proskauer

y = Bright Yellow

β = Beta

+ = Positive reaction

- = Negative reaction

w = Weak reaction

LIST OF ABBREVIATIONS FOR ANTIBIOTIC STUDIES

I = Indeterminate

MIC = Minimal inhibitory concentration

R = Resistant

S = Sensitive

Am = Ampicillin

C = Chloramphenicol

Cb = Carbenicillin

Cl = Cephalothins

Cm = Clindamycin

Cs = Colistin

E = Erythromycin

Gm = Gentamicin

K = Kanamycin

NN = Tobramycin

P = Penicillin

SO = Oxacillin

Te = Tetracycline

Va = Vancomycin

TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
VITA	iii
LIST OF TABLES	iv
LIST OF ABBREVIATIONS	vii
INTRODUCTION	1
Taxonomy	1
Morphological and Biochemical Characteristics	2
Host Specificity	4
Previous Serological Studies	4
Computer Analysis	5
DNA Base Composition and DNA Homology	6
Pathogenicity	8
Isolation and Identification	9
Biochemical Criteria	9
Previous Serological Studies	10
Antibiograms	11
Purpose of This Study	12
MATERIALS AND METHODS	15
Organisms	15
Media and Tests	16
Antibiotic Susceptibility Testing	23
Preparation of Antigen	24
Immunization	24
Tube Agglutination Tests	25
Indirect Fluorescent Antibody Stains	26
Microscopy	28
Adsorptions	28
RESULTS	30
Biochemical Characteristics	30
Weaver Method	30
API Method	38
Antibiotic Susceptibility Patterns	45
Tube Agglutination Studies	53
Indirect Fluorescent Antibody Stains	61
Adsorption Studies	64

Page

DISCUSSION 85

SUMMARY 99

LITERATURE CITED 102

INTRODUCTION

Elizabeth O. King (29) first described the Group IIk organisms as nonfermentative gram negative bacilli which are oxidase and catalase positive, indole negative, motile by means of one polar flagellum, and are also capable of metabolizing carbohydrates by oxidation. King separated Group IIk into two biotypes, IIk-1 and IIk-2, based on their urease reactions and ability to grow on MacConkey agar (IIk-1 negative and IIk-2 positive for both characteristics). A number of workers have attempted to classify these organisms into either the genus Xanthomonas or Pseudomonas by various methods and have discussed their pathogenicity and clinical identification. The purpose of this thesis was to study the biochemical characteristics, antibiograms, and antigenic structure of Groups IIk-1, IIk-2, and possible related bacteria and to attempt to develop a simple, practical method for their identification.

A. TAXONOMY

In the past, most of the chromogenic phytopathogenic bacteria have been classified in the genera Pseudomonas or Xanthomonas of the order Pseudomonadales. The genus Xanthomonas was first described as a nonsporeforming

gram negative bacillus, either motile or nonmotile by means of one (rarely two) polar flagellum (14). All strains tested failed to produce acid from salicin and most strains digested starch and produced acid from lactose. It was also noted that *Xanthomonas* produces a characteristic yellow slimy "mass" on nutrient agar and on potato agar. The production of a slimy yellow growth on sugar containing media, which is evident at both 25 C and 37 C incubation, thus became the distinguishing characteristic of the genus. However, several achromogenic species have subsequently been assigned to the genus *Xanthomonas*, and nonpigmented mutant strains of normally yellow *Xanthomonas* spp. have also been encountered (37). Whether these colorless xanthomonads were naturally occurring or were artificially induced, a dilemma has arisen with the classification scheme due to too great of a reliance upon a single determinative trait.

1. *Morphological and biochemical characteristics*

Early studies concentrated on morphological and biochemical characteristics in an attempt to establish *Xanthomonas* as a separate genus of the order *Pseudomonadales* and also to differentiate *Xanthomonas* species. Starr and Stephens (37) attempted to show that the characteristic pigment is a carotenoid unique to all the members of the genus and lacking in nonxanthomonad bacteria. When they

eluted the pigment from a methanol extract and examined it spectrophotometrically, absorption spectra with maxima of 418, 437, and 463 nm in petroleum ether, 435, 454, and 481 nm in benzene, and 420, 441, and 468 nm in methanol were obtained. None of the yellow nonxanthomonads contained this carotenoid, nor did a number of "incorrectly" designated *Xanthomonas* spp. They suggested that demonstration of these distinctive carotenoids in an organism is sufficient evidence to warrant inclusion in the genus *Xanthomonas*. Moreover, they felt that there are only a limited number of true species rather than the 60 plus species presently recognized.

Subsequently, Hayward (26) studied the biochemical patterns of this genus and found that the organisms were characterized by the following: failure to produce nitrite from nitrate, to decompose nitrite, to produce indole or acetoin, to produce urease, to hydrolyze sodium hippurate, and to produce a UV-fluorescent, diffusible pigment, production of catalase, hydrolysis of esculin, negative or weakly positive oxidase by Kovac's method, and strong lipolytic activity. *Xanthomonas* was reported to be variable for the following characteristics: proteolytic activity, starch hydrolysis, carbohydrate utilization, pectin and pectic acid hydrolysis, production of a brown pigment on media containing tyrosine, and colony morphology.

2. Host specificity

Although the genus *Xanthomonas* is defined on the basis of morphology, pigment production, and biochemical patterns, species identification has been based solely on host specificity (6, 8, 13, 14, 26). This has resulted in the designation of at least 60 species of *Xanthomonas* (13). It is clear, however, that host specificity can not be considered a satisfactory criterion of itself. For example, Dye (13) reported that progressive disease of the bean could be induced by 20 "species" of *Xanthomonas* after four successive passages through bean plants.

3. Previous serological studies

In 1947, Elrod and Braun (14, 15, 16) undertook an extensive serological study of the xanthomonads in order to determine whether antigenic characteristics could be correlated with host range. Initial agglutination experiments revealed a large number of unilateral relationships among strains of xanthomonads due to the abundance of a polysaccharide "gum" produced when the organisms were grown on a 1-2% glucose medium. If the cells were extracted with warm saline, washed, and resuspended, the unilateral relationship was no longer evident (14). Agglutination studies utilizing such saline extracted cells revealed five definite serological groups (14). Following adsorption, all of the organisms in the *X. phaseoli* group showed serological identity but the remaining groups

exhibited a multitude of group specific factors (15, 16). Serologically identical strains (*X. begoniae* and *X. cucurbitae*), for example, were found to infect different hosts (begonias and pumpkins respectively), while some strains (*X. phaseoli* and *X. phaseoli v. sojense*) were found to be capable of attacking the common bean but showed no antigenic similarity (16). Therefore, Elrod and Braun (16) concluded that the antigenic properties vary independently with the ability to infect a given host.

4. Computer analysis

Colwell and Liston (6) applied computer analysis utilizing the Adansonian principle to study the taxonomic position of the xanthomonads. This method had previously been used successfully to differentiate the genera *Enterobacteriaceae*, *Pseudomonadaceae*, and *Bacillaceae* and had been shown capable of separating the species and strains within the genus *Pseudomonas*. The majority of pseudomonads and xanthomonads studied could be classified into three major groups: (i) *Xanthomonas*, (ii) *P. aeruginosa-P. fluorescens*, and (iii) *P. solanacearum*. In the *Xanthomonas* group, two subgroups, *Xanthomonas* I and *Xanthomonas* II, were apparent. *Xanthomonas* I showed a significant relationship with the other pseudomonads, particularly *P. fluorescens*. Two subgroups, Ia and Ib, were identified within *Xanthomonas* subgroup I. Colwell and Liston (6) concluded that *Xanthomonas* is taxonomically distinct from *Pseudomonas*

and that the genus *Xanthomonas* contains two major subgeneric groups. Most of the "species" of *Xanthomonas* appeared, in fact, to fall within one of these two subgroups, thereby suggesting that they constitute a single species.

More recently, King (29) studied a number of xanthomonad- or pseudomonad-like bacteria isolated from human sources assigning them the designation Group IIk and dividing them into biotypes 1 and 2. The work of Von Graevenitz (41) and Tatum et al. (38) subsequently supported this classification. A number of these human isolates were submitted to a numerical taxonomic analysis by Holmes et al. (27). The IIk-1 organisms formed a homogeneous cluster at a 92.5% similarity level and had an average within-taxon similarity of 93.4% which was within the range of values determined for the other taxa studied. The IIk-2 bacteria showed a 100% within-taxon similarity, however, only two strains were tested. Although the IIk-2 and IIk-1 organisms exhibited a high similarity (81.6%), it was concluded that they do not belong in the same group.

5. DNA base composition and DNA homology

The "nomenspecies" concept of bacterial classification, with its latin binomial nomenclature, does not represent a true biological unit. Taxonomists have, therefore, investigated the possibility of a "genospecies" method of classification based on DNA base composition and DNA homology (7, 8, 20). Several investigators have attempted

to apply these methods to the genus *Xanthomonas*.

DeLey and Van Muylem (7) determined the DNA base composition (G+C) of several strains and found that it ranges from 66.0 to 68.2%. They suggested that *Xanthomonas* comprises a narrow group of closely related strains, possibly representing only one or a few "genetic species" (7). In order to check the aforementioned hypothesis, Friedman and DeLey (8, 20) studied the formation of DNA hybrids between these strains. Hybrids were first isolated utilizing the incorporation of heavy isotopes (Deuterated, ^{15}N-labeled DNA), CsCl density-gradient centrifugation method, and it was found that hybrids were readily formed between the DNA of 4 of the 9 strains studied and $D^{15}N$-DNA from *X. pelargonii* P121 (20). Using the more sensitive DNA-agar column method, DeLey and Friedman (8) found that hybridization was on the order of 80-100% for the same species of *Xanthomonas*. Since the base sequences in the chromosomes of the nine strains studied were nearly identical, DeLey et al. (9) proposed that they be considered as one "genospecies" with the name *Xanthomonas campestris*. Subsequent hybridization experiments were performed by the same workers to compare the DNA homology between 28 species of *Xanthomonas* and *Pseudomonas fluorescens* (9). Forty-five to 73% of *P. fluorescens* DNA was found to be identical to that of *Xanthomonas*. The xanthomonads shared almost as much DNA with *P. fluorescens* as did *P. aeruginosa*

and *P. geniculata*. They suggested, therefore, that there is no reason to maintain the genus *Xanthomonas* and proposed that the organisms be reclassified as *Pseudomonas campestris* (9).

Holmes et al. (27) performed DNA base composition studies on 29 strains of Group IIk-1 and on 2 strains of IIk-2. The IIk-1 G+C values ranged from 64.2 to 66.9% with a mean of 65.3%. These values fall within the range defined by Doudoroff and Palleroni (10) for the genus *Pseudomonas* (58 to 70%). When Holmes et al. (27) compared the pigment of the IIk-1 organisms to that of *X. campestris*, they found slight differences in absorbance spectra of the two pigments but the taxonomic significance was uncertain as only a few strains of IIk-1 and *Xanthomonas* were examined. Based upon the fact that the G+C values of the IIk-1 bacteria overlapped the range of the *Pseudomonas* organisms, they proposed the inclusion of the IIk-1 isolates in the genus *Pseudomonas*. They also suggested the species name *paucimobilis*, derived from the group's characteristically sluggish motility.

B. PATHOGENICITY

Although a great deal of work has been done in order to clarify the taxonomic position of Groups IIk-1 and IIk-2, very little is known about their pathogenicity in man. The phytopathogenicity of the xanthomonads has been well documented in a wide variety of plants suffering from severe

necrotic disease (14, 20, 37). Group IIk-like organisms have also been isolated from natural and hospital environmental sources such as water, plants, flow bottles, and ultrasonic nebulizers (30, 41). Although they have been found in human specimens, most commonly from the blood and respiratory tract but also from wound, cervical, and body fluid cultures, the organisms appeared to be etiologically insignificant (25, 27, 35, 40, 41).

C. ISOLATION AND IDENTIFICATION

1. Biochemical criteria

In the past, the identification of the nonfermentative, gram negative rods in clinical laboratories has depened mainly upon a large number of biochemical characteristics. Although this system has been relatively successful in differentiating these bacteria, it requires a large regimen of tests and may take up to 7 days to complete. At present, moreover, there is no general agreement as to what criteria should be used to distinguish Group IIk from the other nonfermenters, particularly the pseudomonads. Pickett and Manclark (33) and Gilardi (24), for example, consistently refer to the IIk bacteria as *Xanthomonas*. In contrast, von Graevenitz (39) differentiates *Xanthomonas* from the IIk group by the oxidase reaction, and IIk-1 from IIk-2 on the basis of growth on MacConkey agar. Oberhoff et al. (31) differentiate IIk-1 from IIk-2 on the basis of the urease reaction (IIk-1 is urea -) and the ability of IIk-2 to

alkalinize allantoin. Weaver et al. (42) distinguished the IIk organisms from the other nonfermentative rods by their ability to oxidize 1% glucose, sucrose, lactose, maltose, and xylose (Leifson's medium), to hydrolyze esculin and to produce oxidase and β-galactosidase. They indicated that the IIk bacteria had uniformly negative reactions for the production of ornithine and lysine decarboxylase, indole, and phenylalanine deaminase, and for the oxidation of mannitol and gluconate.

More recently, Holmes et al. (27) described the new species Pseudomonas paucimobilis (IIk-1) and also differentiated it from the IIk-2 organisms. P. paucimobilis was defined on the basis of its partial motility at room temperature, inability to grow on MacConkey agar, accumulation of poly-β-hydroxybutyrate granules, and acid production from ASS (ammonium salt sugar medium) ethanol. The IIk-2 bacteria exhibited the opposite reactions for these tests. Holmes et al. (27) differentiated the Group IIk organisms from the genus Xanthomonas based on the strong positive oxidase reaction of the IIk strains and their ability to grow in the presence of 2,3,5-triphenyltetrazolium chloride (TTC), utilize asparagine, and produce acid from salicin.

2. Previous serological studies

In 1962, Dye (13) summarized the results of all the previous serological data on xanthomonads, which showed

a close relationship between the multitude of proposed species. No reports of any significance have been published since then and there appears to have been no investigations of the immunology of Group IIk. Such studies would certainly appear warranted since serological techniques recently have provided a means for the rapid identification and biotyping of *Pseudomonas aeruginosa* (3). This work was made possible by the development of a proposed set of well-defined standard *P. aeruginosa* strains and commercial antisera under the auspices of the International Subcommittee on *Pseudomonadaceae* and Related Organisms. Serological identification of the other nonfermentative gram negative rods, including Group IIk, will require the same kind of standardization.

3. *Antibiograms*

A number of workers have attempted to use antibiotic susceptibility patterns as an aid in the identification of the nonfermenters, including the xanthomonads. Gilardi (24) tested seven strains of *Xanthomonas* and found them to be 100% sensitive to tetracycline, chloramphenicol, and erythromycin. The xanthomonads were less susceptible to gentamicin, kanamycin, and neomycin, cephalothin, and nitrofurantoin. Holmes et al. (27) found *P. paucimobilis* (IIk-1) to be uniformly sensitive to ampicillin, chloramphenicol, gentamicin, kanamycin, and tetracycline. The IIk-2 organisms were much more resistant to antimicrobial agents.

Although antibiograms may be used to aid in the identification of the gram negative nonfermenters, some workers have recommended caution against over reliance upon this method. Mutations, excessive use of antimicrobials, and episomal transfer of drug resistance may alter susceptibility patterns (23). It has also been suggested that this method is not specific enough to distinguish between strains (3).

D. PURPOSE OF THIS STUDY

The increasing frequency of human infection with nonfermentative gram negative rods has been clearly documented in the past several years. Although the human pathogenicity of the IIk organisms has not been proven, the increase in their isolation rate, particularly from blood cultures, is alarming. Therefore, a rapid and accurate method of identification was deemed necessary in order to assess the role of these organisms in infectious processes.

Until recently, most microbiology laboratories have used the scheme described by Weaver et al. (42) to identify Group IIK organisms isolated from clinical specimens. This included initial differentiation of the two biotypes based upon growth on MacConkey agar and urease production. These biochemical tests, which are both time consuming and subject to error due to laboratory manipulation, often yield variable results and lead to difficulties in identification.

It was decided, therefore, to explore the possibility of utilizing other modes of identification for the Group IIk organisms. The API 20 E system was selected as an alternative method of identification because it is relatively easy to perform and provides a means to do 20 biochemical determinations in a short period of time (24-48 h). An extensive computerized listing, the API Profile Register, is also available for comparison of the determinations with those of previous workers for IIk isolates.

Since little information was available regarding the antibiograms of the Group IIk organisms, we also decided to carry out the standardized Kirby-Bauer method of antibiotic susceptibility testing on all of our strains of IIk-1 and IIk-2 in order to determine whether they had any characteristic patterns. Kirby-Bauer susceptibility patterns are routinely performed in clinical laboratories and, therefore, could possibly be an important tool in the identification of Group IIk.

Since serological typing had recently been used so successfully to identify strains of _P. aeruginosa_, we attempted to apply a similar approach to the identification of IIk. Antisera were prepared against several strains of IIk-1 and IIk-2 and were tested against homologous and heterologous organisms utilizing both agglutination and indirect fluorescent antibody staining techniques. The specificity of the reactions was assessed by adsorption

studies utilizing homologous and heterologous controls.

The primary purpose of this study was to develop reliable criteria for the identification of Group IIk and to evaluate the methods developed in the course of this work for the rapid identification of the Group IIk organisms isolated from clinical specimens.

MATERIALS AND METHODS

A. ORGANISMS

Eight strains of Group IIk were supplied by Dr. R. E. Weaver from the Center for Disease Control, Atlanta, Georgia: (a) C 1196 IIk-1; (b) B 9042 IIk-2; (c) B 8684 IIk-2; (d) C 198 IIk-2; (e) C 1279 IIk-2; (f) C 1822 IIk-2; (g) C 499 IIk-2; and (h) C 486 IIk-2. Dr. G. L. Gilardi from the Hospital for Joint Diseases Medical Center, New York, New York provided 10 strains of the IIk organism (submitted as *Xanthomonas* sp.): (a) 1614G; (b) 2232G; (c) 1854G; (d) 2146G; (e) 2123G; (f) 2147G; (g) 1852G; (h) 2148G; (i) 1897G; and (j) 1672G. Two strains of IIk-1 (a) K 1 (cervix) and (b) K 2 (wound) were supplied by Dr. F. Kocka at Edgewater Hospital, Chicago, Illinois and 17 additional strains of IIk were provided by API (Ayerst Laboratories) in Plainview, New York: IIk-1 strains (a) 76-934; (b) 76-941; (c) 76-942; (d) 76-933; (e) 76-940 and IIk-2 strains (a) 76-944; (b) 76-949; (c) 76-951; (d) 74-1543; (e) 76-950; (f) 76-952; (g) 76-946; (h) 76-947; (i) 74-1545; (j) 76-943; (k) 76-945; and (1) 74-1540. *Pseudomonas aeruginosa* ATCC 10145, *Pseudomonas cepacia* ATCC 10856, *Pseudomonas maltophilia* ATCC 13637, *Pseudomonas fluorescens* ATCC 13525, *Flavobacterium meningosepticum* ATCC 13253, *Acinetobacter calcoaceticus* ATCC 23055, and

Xanthomonas campestris ATCC 6402 were obtained from the American Type Culture Collection. Four additional strains of IIk were isolated from clinical specimens from the laboratory at Foster G. McGaw Hospital of Loyola University: (a) LMC 1 IIk-1 (wound); (b) LMC 2 IIk-1 (ear); (c) LMC 3 IIk-2 (wound); and (d) LMC 4 IIk-1 (blood).

B. MEDIA AND TESTS

Trypticase soy agar plates with 5% defibrinated sheep red blood cells (BBL) were used for the isolation of all organisms. The plates were incubated at 35 C in a 5% CO_2 atmosphere. P. fluorescens ATCC 13525 and X. campestris ATCC 6402 were incubated at 25 C since there was little or no growth at 35 C. Although increased carbon dioxide tension was not an absolute requirement for the growth of these organisms, it did enable us to obtain larger yields for subsequent biochemical studies. Biochemical tests for P. fluorescens ATCC 13525 and X. campestris ATCC 6402 were incubated at 25 C; all others were incubated at 35 C in air unless otherwise stated. All tests were performed at least twice. When discrepancies occurred the tests were repeated at least once and the result obtained most often was recorded.

The oxidation tests were performed on OF basal medium with 10% added carbohydrate (18, 28). Control tubes without carbohydrate were used with each test. The transfer broth used to inoculate the carbohydrates was

2.5 ml of trypticase soy broth (Difco). The growth obtained from 48 h cultures on blood agar plates was transferred to the trypticase soy broth (TSB) with a Dacron Swab (Scientific Products) to make a heavy suspension. The oxidation tubes were then inoculated with approximately 0.3 ml of the bacterial suspension by stabbing with a pasteur pipet and releasing the suspension slowly while withdrawing the pipet from the medium.

The oxidase test was performed by placing a loopful of a culture grown on sheep blood agar onto a strip of filter paper and adding a few drops of 1% aqueous tetramethyl-p-phenylenediamine dihydrochloride (38). A test was considered positive if the organism turned purple within 10 sec.

Catalase production was tested for by placing a drop of 3% H_2O_2 on a glass slide, adding a loopful of the organism, and checking for the evolution of gas bubbles (32).

Growth on MacConkey agar was detected by inoculating MacConkey agar slants (Difco) with a six-hour TSB culture and incubating at 35 C (24). The slants were checked for growth at 24 and 48 h.

ONPG disks (Difco) were used to test for the presence of β-galactosidase according to the manufacturer's instructions.

Six-hour TSB cultures were used to inoculate tubes

of TSI, urea, citrate, and tryptone. TSI, urea, and citrate reactions were checked at 24 and 48 h. TSI agar results were recorded in the following manner: slant (lactose and sucrose fermentation), butt (glucose fermentation), gas from glucose and H_2S production (17). A positive citrate was signified by a change in color of the slant from green to blue (36). The presence of urease was determined by the organism's ability to produce an alkaline environment (denoted by a pink to red color) in Christensen's medium (17). Since organisms other than Proteus may produce various degrees of alkalinitity, reactions were rated as 1-4+. A 1+ urease showed a pink color on half of the agar slant, whereas, an entirely pink slant was considered a 2+. A 3+ urease signified a color change for the slant and half of the butt, while a pink to red color throughout the medium was considered a 4+. Xylol and Ehrlich's reagent were added to the tryptone broth after 48 h of incubation to test for indole production (38). The test was considered positive if a pink to red color developed in the xylol layer.

Motility was tested with Difco's Motility Test Medium by stabbing the agar with an inoculating needle and incubating at 25 C (17). The tubes were checked at 24 and 48 h.

Bile esculin slants were inoculated with a heavy TSB suspension. Production of a black pigment in 24 to

48 h indicated a positive test for esculin hydrolysis (24).

To detect gelatinase production, a heavy suspension of organisms was made in 1 ml of distilled water. A gelatin strip (Key Scientific) was added to the tube and incubated according to the manufacturer's instructions. A change in color of the strip from green to blue after 6 h was considered positive.

Nitrate and nitrite reduction were determined by the reactions of the organisms on fluorescence-denitrification agar slants (F/N slants, Key Scientific) and in nitrate broth (Pfizer). The presence of gas bubbles in the butt of the F/N slant showed the ability of the organism to reduce NO_3 or NO_2 to N_2 gas after 48 h of incubation (34). One drop of 6 N HCl was added to a 48 h nitrate broth culture followed by 12 drops of nitrite test reagent (Key). The development of a pink color was considered to be a positive test for NO_2 production (32). Zinc dust was added to all negative tubes and, if a pink color developed, the test was reported as negative since the zinc was responsible for reducing the unutilized NO_3. If the addition of zinc dust did not result in the production of a pink color, the test was considered positive for the reduction of NO_3 to N_2 gas (32).

F/N slants were also used to detect the presence of auto-fluorescence. After 48 h of incubation the tubes were placed under a UVSL·25 Mineralight in a dark room and

checked against a positive (*P. fluorescens*) and negative (*P. cepacia*) control (34). Positive organisms fluoresced under the UV lamp.

The gluconate test (Key) was performed by inoculating the substrate heavily and incubating for 24 h. A Clinitest tablet (Ames) was then added according to the manufacturer's instructions to detect the presence of reducing sugars. A negative test was blue and a positive ranged from green to dark orange.

Growth on Salmonella-Shigella agar (SS) was tested for by streaking a heavy inoculum of the organism on SS plates (BBL) and observing the media for growth after 24 and 48 h of incubation (19).

Phenylalanine slants (Difco) were used to detect the production of phenylalanine deaminase (32). The slants were inoculated with a 6 h broth culture and incubated for 24 h. Several drops of 10% (w/v) ferric chloride solution were added to the slants which were then examined for the development of a green color in the agar.

Organisms from 24 h blood agar cultures were stained with Sudan black to determine the accumulation of (PBHB) poly-β-hydroxybutyrate (4, 19). Three tenths gram of Sudan black B were dissolved in 100 ml of 70% alcohol and then allowed to age overnight. A thin smear of the organism was made and heat fixed. The smear was flooded with Sudan black for 10 min, drained, and blotted

dry. Xylol was used as a clearing agent and then the slide was counter-stained with 0.5% aqueous safranin. Any PBHB granules present in the cells stained black while the rest of the bacteria stained pink. Smears were examined microscopically using oil immersion (1000 X).

The presence of deoxyribonuclease (DNAse) was tested for by placing a heavy inoculum of the organisms on DNAse test agar with toluidine blue (BBL) (24). The DNAse plates were incubated for 48 h. If a pinkish-red complex formed around the colonies, the test was considered to be positive.

Both lecithinase and lipase activity were determined by heavily inoculating trypticase soy agar (TSA) plates supplemented with 10% egg yolk enrichment and incubating for 48 h (24). If lecithinase was present, a white precipitate formed around the streaks of growth. Lipase activity was detected by observing the colonies and surrounding medium for an irridescent sheen.

Casein hydrolysis was demonstrated by growing the bacteria on TSA plates with 10% powdered skim milk for 48 h (24). A positive test was signified by the presence of a clear zone around the colonies.

Growth at 42 C was observed by lightly inoculating brain heart infusion agar slants (BBL) and 2.5 ml of TSB which had been preincubated at 42±0.5 C (24). The inoculated tubes were examined after 24 h of incubation.

The buffered substrate system of Key Scientific was

chosen to test for lysine and ornithine decarboxylase activity because it does not require active growth of the organism as does the Moeller decarboxylase broth. The Key tablets are made to detect the presence of the enzymes, whereas the Moeller medium detects the end products of their activity in a tube covered with sterile mineral oil or paraffin. The nonfermenters do not grow well in an atmosphere that is not rich in oxygen and, therefore, do not give consistent results with the Moeller medium. Lysine and ornithine decarboxylase activity were detected by placing two loopfuls of the organisms into 1 ml of distilled water and then adding a lysine or ornithine tablet as instructed by the manufacturer. After 24 h of incubation, two drops of 40% KOH and 1 ml of ninhydrine in chloroform were added to the tubes which were then allowed to stand at room temperature for 10 min. A positive test was indicated by a color change in the chloroform layer, violet for lysine and pink for ornithine.

The API 20 E system (Ayerst Laboratories) is a strip of plasticized paper consisting of twenty microtubes containing dehydrated media which are basically identical to the media used in conventional identification schemes. The biochemicals included on the strip were originally selected to identify the Enterobacteriaceae and other fermenting gram negative rods. The media in the microtubes was reconstituted by adding a saline suspension (10^6 bacteria/ml)

of the test organisms according to the manufacturer's instructions. The strips were then incubated at 35 C for 48 h. The twenty biochemical determinations available on the strip are: ONPG, citrate, urease, V-P, arginine dihydrolase, H_2S, indole, gelatin, tryptophan deaminase, glucose, sucrose, amygdalin, mannitol, inositol, sorbitol, rhamnose, melibiose, arabinose, and ornithine and lysine decarboxylase. API has recommended the addition of six tests, utilizing conventional media, to aid in the identification of the nonfermentative rods. These tests are: oxidase, nitrate reduction, motility, growth on MacConkey agar, and oxidation and fermentation of glucose.

After reading the biochemical reactions on the API 20 strip a profile number for the organism was obtained. These profile numbers were compared with those in the API Profile Register in an attempt to identify the organisms. API continually updates their computerized list of profile numbers as new biochemical patterns are discovered.

C. ANTIBIOTIC SUSCEPTIBILITY TESTING

Antibiotic susceptibility testing was performed according to the single disk diffusion method described by Bauer et al. (2). All susceptibility tests were performed at least twice. If discrepancies occurred the tests were repeated at least once and the result obtained most often was recorded.

D. PREPARATION OF ANTIGEN

The IIk organisms were inoculated onto brain heart infusion agar plates (BHIA) and incubated at 35 C for 48 h. The bacteria were washed off the BHIA with 10 ml of 0.5% formalin and incubated at 35 C for 72 h. The suspensions were then tested for viability by plating a loopful of the bacteria-formalin mixture onto another BHIA and incubating at 35 C for 48 h.

Bacteria were washed five times with 0.85% saline containing 0.025% formalin and 0.01% sodium azide. The bacterial suspensions were adjusted to a density equivalent equal to a no. 7 McFarland standard (2.1×10^9 bacteria/ml) in 0.85% saline and then used for inoculations.

E. IMMUNIZATION

New Zealand white rabbits weighing 2 to 2.5 kg were inoculated according to the following schedule: day 1, 0.25 ml of the cell suspension incorporated in 0.25 ml of Freund's complete adjuvant (Difco) was injected intradermally and subcutaneously into several sites of the footpads and back; day 7, 0.1 ml of the cell suspension incorporated in 0.1 ml of Freund's complete adjuvant was injected intramuscularly into each thigh; and day 21, 1.0 ml of the cell suspension was injected intravenously into the ear. The rabbits were bled from the marginal ear vein on day 28 and every 2 to 3 days thereafter for one week. The blood was centrifuged at 2500 rpm until all red blood cells were

removed. The sera collected from all bleedings of an individual rabbit were than pooled and frozen in 1.0 ml aliquots at -50 C.

A total of thirty-five rabbits were immunized. The antisera were designated according to the antigen used for the immunizations (e.g. anti-76-947 was prepared by immunization of a rabbit with IIk strain 76-947). Each antiserum was first tested for activity against its respective antigen by slide agglutination tests. A 3-4+ agglutination was obtained for each antiserum. A saline control was run with each organism to test for self-agglutinating strains but none were found.

F. TUBE AGGLUTINATION TESTS

O antigen was prepared by growing the organisms on BHIA at 35 C for 48 h. The growth was washed from the plates with 0.85% sterile saline and the organisms were washed 3 times in 10 ml aliquots of 0.85% saline. Following centrifugation (GLC-1 Sorvall) at 2500 rpm for 15 min, the supernatant was decanted and the packed cells were resuspended in 10 ml of saline and boiled for 1 h. The heat-killed cells were washed and resuspended in saline, adjusting the turbidity to that of a no. 3 McFarland standard (9×10^8 bacteria/ml). This suspension was used in the agglutination studies.

To perform the tube dilutions, 0.9 ml of saline was pipetted into the first of ten 13 x 100 mm test tubes and

0.5 ml of saline were added to the remaining nine. One tenth ml of the antiserum was placed into the first tube and mixed thoroughly. With a new pipet, 0.5 ml of the 1:10 dilution was transferred to the second tube and a two-fold serial dilution was performed until a final dilution of 1:2560 was obtained (tube 8). The ninth tube was a saline control and the tenth tube contained 0.5 ml of a 1:10 dilution of normal rabbit serum. Five tenths ml of the organism suspension was then added to each tube. All tubes were then incubated for 18 h at 35 C and examined for agglutination (5). The endpoint titer was the last tube showing a 2+ agglutination. Agglutination reactions were rated according to the following gradations: 1+ (25%) - questionable clumping; 2+ (50%) - definite clumping, supernate cloudy; 3+ (75%) - definite clumping, supernate clearing; and 4+ (100%) - completely precipitated, supernate clear (18).

G. INDIRECT FLUORESCENT ANTIBODY STAINS

Fluorescent antibody testing was carried out according to the method outlined below: O antigen was prepared by growing the organisms on BHIA for 48 h. The growth was washed from the plates with 0.85% saline and the organisms were washed three times as previously described. After decanting the supernatant, the bacteria were resuspended in 10 ml of sterile saline and boiled for 1 h. After boiling, the organisms were centrifuged

again and the supernatant discarded. Saline was used to resuspend the organisms.

A loopful of the antigen suspension was spread on alcohol washed slides and allowed to air dry. The antigen was then fixed to the slides by placing them in 95% ethanol for 1 min. The slides were washed in FTA Hemagglutination Buffer (BBL) for 5 min and air dried. If the slides were not used immediately they were frozen for up to a week at -50 C.

The antigen smears were overlayed with a 1:10 dilution of antiserum and incubated in a moist chamber at 37 C for 30 min. Normal rabbit serum and saline were used as controls. Excess antiserum was removed from the slides by rinsing in a stream of FTA buffer. The slides were then soaked in two changes of FTA buffer for 5 min and air dried. A 1:40 dilution of fluorescein-conjugated goat anti-rabbit globulin (BBL) was overlayed on the smears and the slides were again incubated at 37 C for 30 min. A saline-conjugate smear was used as a conjugate control. The smears were rinsed and soaked as described before and air dried. A drop of buffered-glycerol (BBL) was placed on each smear and mounted with a coverslip. The slides were read on an AO Spencer microscope and fluorescence was rated from 0 to 4+. Only reactions of 2+ or greater were considered positive. The intensity of the apple green fluorescence was rated subjectively based on the memory and experience of

the investigator. All tests were evaluated by two investigators reading the slides independently and then comparing the results. A total lack of fluorescence was rated as 0 and increasing intensities were rated from 1-4+ with 4+ as the maximum. Titers of the antisera were determined by performing standard two-fold dilutions and overlaying separate smears with each of the serum dilutions. The staining technique was carried out as previously described.

H. MICROSCOPY

Microscopy was performed with an AO Spencer microscope, model 645, equipped with an Osram HBO 200 high pressure mercury lamp and a darkfield condenser for oil immersion. The following filters were used: A Corning 702 exciter filter with a yellow barrier filter in combination with a yellow-orange barrier filter. A 10X ocular lens and a 45X objective lens were used for magnification.

I. ADSORPTIONS

Bacteria were grown on BHIA and incubated at 35 C in 5% CO_2 for 48 h. The bacteria were rinsed from the plates in 0.85% saline and subsequently washed three times as described above. The organisms were boiled for 1 h, centrifuged, and the supernatant discarded. One ml of packed, washed bacteria was mixed with 0.5 ml of antiserum diluted 1:5 with saline. The mixture was incubated at 35 C for 2 h followed by overnight incubation at 4 C. The adsorptions were done at least twice or until a negative agglutination

reaction occurred when the adsorbed antiserum was tested with the adsorbing antigen. The adsorbed antisera were then used in agglutination studies in order to assess the specificity of the previous serological reactions.

RESULTS

A. BIOCHEMICAL CHARACTERISTICS

1. Weaver Method

Tables 1 through 4 summarize the results of the biochemical tests performed on all 41 strains of IIk and the 7 control ATCC organisms. The strains of IIk utilized in this study showed a high degree of similarity in their biochemical characteristics based on the 33 tests used in the conventional Weaver system of identification. All IIk strains exhibited positive reactions for the following tests: ONPG, hydrolysis of esculin, and oxidation of dextrose, xylose, sucrose, maltose, and lactose. Only one strain (LMC 1) was negative for oxidase production and one (74-1543) gave a negative catalase reaction. Negative reactions were obtained for the characteristics listed below for all IIk organisms: gluconate and mannitol oxidation; gelatinase, phenylalanine deaminase, lipase, lecithinase, and indole production; decarboxylation of lysine and ornithine; growth on SS agar; and auto-fluorescence.

Although Weaver et al. (42) distinguished between IIk-1 and IIk-2 on the basis of the urease reaction and growth on MacConkey agar, only 92% (22/24) of our IIk-2 strains gave positive urease reactions and only 88% (21/24) grew on MacConkey agar (Table 3), while 24% (4/17) of the

TABLE 1a

Biochemical Reactions of 41 Strains of Group IIk Utilizing the Conventional Weaver Method of Identification

Organisms	Tests or Substrates														
	Oxidase	Catalase	Pigment	TSI	Urease	MacConkey	Motility	PBHB	42 C TSB/BHI	Rhamnose	Nitrate	DNAse	Citrate	Casein	Hemolysis
IIk-2[a]															
76-950	+	+	-	AK--[b]	3+	+	-	-	-/-	-	-	-	-	-	-
76-940	+	+	-	AK--	4+	+	-	-	-/-	-	-	-	-	-	-
76-944	+	+	-	AK--	4+	+	-	-	-/-	-	-	-	-	-	-
74-1543	+	-	-	AK--	4+	+	-	-	-/-	-	-	-	-	-	-
76-947	+	+	ly	AK--	4+	+	-	-	-/-	-	-	-	-	-	-
76-943	+	+	-	AK--	4+	+	-	-	-/-	-	-	-	-	-	-
C 486	+	+	-	AK--	4+	+	-	-	-/-	-	-	-	-	-	-
2147G	+	+	-	AK--	4+	-	-	-	+/-	A	-	-	-	-	-
2148G	+	+	-	AK--	4+	+	-	-	+/-	A	-	-	-	-	-
C 1822	+	+	-	AK--	3+	+	-	-	-/-	-	-	-	-	-	-

TABLE 1b (cont'd)

Organisms	Tests or Substrates														
IIk-2[a]	Oxidase	Catalase	Pigment	TSI	Urease	MacConkey	Motility	PBHB	42 C TSB/BHI	Rhamnose	Nitrate	DNAse	Citrate	Casein	Hemolysis
C 198	+	+	ly	KK--	4+	+	-	-	-/-	-	-	-	-	-	-
76-945	+	+	-	AK--	4+	+	-	-	-/-	-	-	-	-	-	-
LMC 3	+	+	-	AK--	4+	+	-	-	-/-	-	+	-	-	-	β
2146G	+	+	ly	KK--	1+	+	-	-	-/-	-	-	+	-	-	-
74-1540	+	+	-	AK--	4+	+	-	-	-/-	-	-	-	-	-	-
76-946	+	+	-	AK--	-	+	-	-	+/+	-	-	-	-	-	-
74-1545	+	+	-	KK--	-	-	-	-	+/+	-	-	-	-	-	-
C 499	+	+	-	AK--	4+	+	-	-	+/+	A^{W}	+	+	-	-	-
76-951	+	+	ly	AK--	4+	+	-	-	+/+	A^{W}	-	+	-	+	-
B 9042	+	+	-	AK--	4+	+	-	-	-/-	-	-	-	-	-	-
76-952	+	+	-	AK--	3+	+	-	-	-/-	-	-	-	-	-	-
B 8684	+	-	ly	KK--	4+	-	-	-	+/-	-	+	-	-	-	-

TABLE 1c (cont'd)

Organisms	Oxidase	Catalase	Pigment	TSI	Urease	MacConkey	Motility	PBHB	42 C TSB/BHI	Rhamnose	Nitrate	DNase	Citrate	Casein	Hemolysis
	Tests or Substrates														
IIk-2[a]															
C 1279	+	+	-	AK--	4+	+	-	-	-/-	-	-	-	-	-	-
76-949	+	+	-	AK--	4+	+	-	-	-/-	-	-	-	-	-	-
IIk-1[a]															
1614G	+	+	Y	KK--	-	-	+	-	-/-	-	-	-	-	-	-
2123G	+	+	Y	KK--	3+	-	+	+	-/-	-	+	-	-	-	-
76-934	+	+	Y	KK--	-	-	+	+	+/-	-	+	-	-	-	-
76-941	+	+	Y	KK--	-	-	+	-	-/-	-	-	-	-	-	-
76-942	+	+	Y	KK--	-	-	+	+	-/-	-	-	-	-	-	-
1672G	+	+	Y	KK--	-	-	+	-	-/-	-	-	-	-	-	-
IMC 4	+	+	Y	KK--	-	-	+	+	-/-	-	-	-	-	-	-
76-933	+	+	Y	KK--	-	-	-	+	-/-	-	-	-	-	-	-
K 2	+	+	Y	KK--	-	-	-	-	-/-	-	-	-	-	-	-

TABLE 1d (cont'd)

Organisms	Tests or Substrates														
IIk-1[a]	Oxidase	Catalase	Pigment	TSI	Urease	MacConkey	Motility	PBHB	42 C TSB/BHI	Rhamnose	Nitrate	DNAse	Citrate	Casein	Hemolysis
LMC 1	-	+	Y	KK--	1+	+[w]	+	-	-/-	-	-	-	+	-	-
LMC 2	+	+	Y	AK--	1+	-	+	+	-/-	-	-	-	-	-	-
1897G	+	+	Y	KK--	1+	+[w]	+	-	+/+	-	-	-	-	-	-
C1196	+	+	Y	KK--	-	+[w]	-	-	-/-	-	-	-	+	-	-
1852G	+	+	Y	KK--	-	-	-	-	-/-	-	-	-	-	-	-
1854G	+	+	Y	KK--	-	-	+	-	-/-	-	-	-	-	-	-
2232G	+	+	Y	KK--	-	-	+	-	-/-	A	-	-	-	-	-
K 1	+	+	Y	KK--	-	+	-	+	-/-	A	-	-	-	-	β

[a]All IIk strains were positive for the following reactions: ONPG; esculin hydrolysis; and dextrose, xylose, sucrose, maltose, and lactose oxidation.

All IIk strains were negative for the following reactions: glucose and mannitol oxidation; lysine and ornithine decarboxylation; gelatinase, lipase, lecithinase, and indole production; growth on SS agar; auto-fluorescence; and phenylalanine deaminase production.

[b]TSI agar was read slant/butt/gas from glucose/H_2S, A = acid, K = alkaline.

TABLE 2

Summary of the Reactions of 17 Strains of Nonfermentative Rod IIk-1 When Tested by Weaver's Scheme

Tests or Substrates	Number Positive	Number Negative	Percent Positive
Oxidase	16	1	94
ONPG	17	0	100
Bile esculin	17	0	100
OF Dextrose	17	0	100
OF Xylose	17	0	100
OF Sucrose	17	0	100
OF Maltose	17	0	100
OF Lactose	17	0	100
OF Rhamnose	2	15	12
OF Mannitol	0	17	0
Catalase	17	0	100
Pigment	17	0	100
Growth on MacConkey agar	4	13	24
TSI (AK--)	1	16	6
TSI (KK--)	16	1	94
Urease	4	13	24
Motility	12	5	71
42 C TSB/BHI	2/1	15/16	12/6
PBHB	7	10	41
Nitrate	2	15	12
DNAse	0	17	0
Casein	0	17	0
Hemolysis	1	16	6
Gelatinase	0	17	0
Phenylalanine deaminase	0	17	0
Indole	0	17	0
Citrate	2	15	12
Ornithine	0	17	0
Lysine	0	17	0
Growth on SS agar	0	17	0
Gluconate	0	17	0
Lipase	0	17	0
Lecithinase	0	17	0
F/N	0/0	17/17	0/0

TABLE 3

Summary of the Reactions of 24 Strains of Nonfermentative Rod IIk-2 When Tested by Weaver's Scheme

Tests or Substrates	Number Positive	Number Negative	Percent Positive
Oxidase	24	0	100
ONPG	24	0	100
Bile esculin	24	0	100
OF Dextrose	24	0	100
OF Xylose	24	0	100
OF Sucrose	24	0	100
OF Maltose	24	0	100
OF Lactose	24	0	100
OF Rhamnose	4	20	17
OF Mannitol	0	24	0
Catalase	22	2	92
Pigment	5	19	21
Growth on MacConkey agar	21	3	88
TSI (AK--)	20	4	83
TSI (KK--)	4	20	17
Urease	22	2	92
Motility	0	24	0
42 C TSB/BHI	7/4	17/20	29/17
PBHB	0	24	0
Nitrate	3	21	13
DNAse	3	21	13
Casein	2	22	8
Hemolysis	1	23	4
Gelatinase	0	24	0
Phenylalanine deaminase	0	24	0
Indole	0	24	0
Citrate	0	24	0
Ornithine	0	24	0
Growth on SS agar	0	24	0
Gluconate	0	24	0
Lipase	0	24	0
Lecithinase	0	24	0
F/N	0	24	0/0

TABLE 4

Biochemical Reactions of ATCC Control Cultures

Using Weaver's Identification Method

Tests or Substrates	*P. aeruginosa* ATCC 10145	*P. fluorescens* ATCC 13525	*P. cepacia* ATCC 10856	*P. maltophilia* ATCC 13637	*F. meningosepticum* ATCC 13253	*A. calcoaceticus* ATCC 23055	*X. campestris* ATCC 6402
Oxidase	+	+	+	+	+	-	+
ONPG	-	-	+	+	+	-	+
Bile esculin	-	-	+	+	+	-	+
OF Dextrose	A	A	A	A^{w}	A	A	A
OF Xylose	A	A	-	-	-	A	A
OF Sucrose	-	A	A	A^{w}	-	-	A
OF Maltose	-	-	A^{w}	A	A	-	A
OF Lactose	-	-	A	A^{w}	A	A^{w}	-
OF Rhamnose	-	-	-	-	-	A	A
OF Mannitol	A	A^{w}	A^{w}	-	A	-	A^{w}
Catalase	+	+	+	+	+	+	+
Pigment	g	-	-	g	-	-	y
Growth on MacConkey agar	+	+	+	+	+	-	-
TSI (KK--)	+	+	+	+	+	+	+
Urease	+	+	+	-	-	-	+
Motility	+	+	+	+	-	-	+
42 C TSB/BHI	+/+	-/-	+/-	-/+	-/-	-/-	-/-
PBHB	-	-	+	-	-	-	-
Nitrate	+	-	-	+	-	-	-
DNAse	-	-	-	+	+	-	-
Casein	+	-	+	+	+	-	-
Hemolysis	+	-	-	-	-	-	-
Gelatinase	+	+	+	+	+	-	-
Phenylalanine deaminase	-	-	-	-	-	-	-
Indole	-	-	-	-	+	-	-
Citrate	+	+	+	+	-	-	-
Ornithine	-	-	-	-	-	-	-
Lysine	-	-	+	+	-	-	-
Growth on SS	+	-	-	$+^{w}$	-	-	-
Gluconate	+	$+^{w}$	-	-	-	-	-
Lipase	-	-	+	+	-	-	-
Lecithinase	$+^{w}$	-	-	-	-	-	-
F/N	-/+	+/-	-/-	-/-	-/-	-/-	-/-

IIk-1 strains were also urease positive and grew on MacConkey agar (Table 2). All of the IIk-1 organisms produced a bright yellow pigment on sheep blood agar whereas only 21% (5/24) of the IIk-2 strains produced a light yellow pigment and this occurred only after prolonged incubation at room temperature. The majority (94% or 16/17) of the IIk-1 strains gave a KK-- reaction in TSI while the majority (83% or 20/24) of the IIk-2 bacteria gave an AK-- reaction in TSI. Variable reactions were obtained for both biotypes for rhamnose oxidation, motility, growth at 42 C, PBHB accumulation, nitrate reduction, casein hydrolysis, hemolysis, DNAse production, and citrate. The ATCC control organisms gave the expected biochemical patterns and could be differentiated from the IIk strains (Table 4).

2. API method

When tested with the API 20 E system, the 41 strains of IIk showed a greater degree of biochemical variability than was observed with the conventional Weaver method. Tables 5 through 7 summarize these results. All IIk strains exhibited negative results for decarboxylation of ornithine and lysine, arginine hydrolysis, H_2S and indole production, nitrate reduction, and oxidation of mannitol, sorbitol, inositol, and rhamnose. The majority of both IIk biotypes gave positive reactions for ONPG, V-P, and citrate. Eighty-eight percent (21/24) of the IIk-2 organisms were positive

TABLE 5a

Biochemical Reactions of 41 Strains of Group IIk Utilizing the API System of Identification

Organisms	Tests or Substrates										
IIk-2*	ONPG	Glucose	Sucrose	Amygdalin	Urease	V-P	Citrate	Gelatin	Melibiose	Arabinose	TDA
76-950	+	+	-	+	+	+	+	-	-	-	-
76-940	+	-	+	+	+	-	+	-	-	-	-
76-944	+	+	+	+	+	+	+	-	-	-	-
74-1543	+	+	+	+	+	+	+	-	-	-	-
76-947	-	+	+	-	+	-	-	-	-	-	-
76-943	-	+	-	+	+	+	+	-	-	-	-
C 486	+	+	+	+	+	+	+	-	-	-	-
2147G	+	+	+	+	+	+	+	-	+	-	-
2148G	+	+	-	+	+	-	+	-	+	-	-

TABLE 5b (cont'd)

Organisms	Tests or Substrates										
IIk-2*	ONPG	Glucose	Sucrose	Amygdalin	Urease	V-P	Citrate	Gelatin	Melibiose	Arabinose	TDA
C 1822	+	+	+	+	+	+	+	-	-	-	-
C 198	+	+	+	+	+	+	-	-	+	-	-
76-945	+	+	+	+	+	+	+	-	-	-	-
LMC 3	+	+	+	-	+	+	-	-	-	+	-
2146G	+	-	+	+	-	+	+	+	-	-	-
74-1540	+	+	+	-	+	+	+	-	-	+	-
76-946	+	+	+	+	-	+	-	-	-	-	-
74-1545	+	+	+	+	-	+	-	-	-	+	-
C 499	+	-	+	+	+	-	+	-	-	-	-
76-951	+	+	+	+	+	-	+	+	-	-	-
B 9042	+	+	+	+	+	+	+	+	-	-	-
76-952	+	+	+	+	+	+	+	-	-	-	-

TABLE 5c (cont'd)

Organisms	Tests or Substrates										
	ONPG	Glucose	Sucrose	Amygdalin	Urease	V-P	Citrate	Gelatin	Melibiose	Arabinose	TDA
IIk-2*											
B 8684	-	+	+	+	+	+	+	-	-	-	-
C 1279	+	+	+	+	+	+	+	-	-	-	-
76-949	+	+	+	+	+	+	+	-	-	-	-
IIk-1*											
1614G	+	-	-	-	-	+	+	-	-	-	-
2123G	+	-	-	-	-	+	+	-	-	-	-
76-934	+	-	-	-	-	+	-	-	-	-	-
76-941	+	-	-	-	-	+	+	-	-	-	-
76-942	+	-	-	-	-	+	-	-	-	-	-
1672G	+	-	+	-	-	+	+	-	-	-	-
LMC 4	+	-	-	-	-	-	+	-	-	-	-

TABLE 5d (cont'd)

Organisms	Tests or Substrates										
IIk-1*	ONPG	Glucose	Sucrose	Amygdalin	Urease	V-P	Citrate	Gelatin	Melibiose	Arabinose	TDA
76-933	+	-	-	-	-	+	+	-	-	-	-
K 2	+	-	-	-	-	+	-	-	-	+	+
LMC 1	+	-	-	-	-	+	-	-	-	-	-
LMC 2	+	-	+	-	-	+	+	-	-	-	-
1897G	+	-	-	-	-	+	+	-	-	-	-
C 1196	+	-	-	-	-	+	-	-	-	-	-
1852G	+	-	-	-	-	-	+	-	-	-	-
1854G	+	-	-	-	-	-	+	-	-	-	-
2232G	-	-	-	-	-	-	+	-	-	-	-
K 1	+	-	-	-	-	+	+	-	-	-	-

*All IIk strains were negative for the following reactions: ornithine and lysine decarboxylation; arginine dihydrolase, indole, and H_2S production; and mannitol, inositol, sorbitol, and rhamnose oxidation.

TABLE 6

Summary of the Reactions of 17 Strains of Nonfermentative Rod IIk-1 When Tested by the API 20 E System

Tests or Substrates	Number Positive	Number Negative	Percent Positive
ONPG	16	1	94
V-P	13	4	77
Glucose	0	17	0
Sucrose	2	15	12
Amygdalin	0	17	0
Citrate	12	5	71
Urease	0	17	0
Gelatin	0	17	0
Melibiose	0	17	0
Arabinose	1	16	6
TDA	1	16	6
Arginine dihydrolase	0	17	0
Lysine decarboxylase	0	17	0
Ornithine decarboxylase	0	17	0
H_2S	0	17	0
Indole	0	17	0
Mannitol	0	17	0
Inositol	0	17	0
Sorbitol	0	17	0
Rhamnose	0	17	0

TABLE 7

Summary of the Reactions of 24 Strains of Nonfermentative Rod IIk-2 When Tested by the API 20 E System

Tests or Substrates	Number Positive	Number Negative	Percent Positive
ONPG	21	3	88
V-P	19	5	79
Glucose	21	3	88
Sucrose	21	3	88
Amygdalin	21	3	88
Citrate	19	5	79
Urease	21	3	88
Gelatin	3	21	13
Melibiose	3	21	13
Arabinose	3	21	13
TDA	0	24	0
Arginine dihydrolase	0	24	0
Lysine decarboxylase	0	24	0
Ornithine decarboxylase	0	24	0
H_2S	0	24	0
Indole	0	24	0
Mannitol	0	24	0
Inositol	0	24	0
Sorbitol	0	24	0
Rhamnose	0	24	0

for urease production (Table 7) while none of the IIk-1 strains gave a positive urease reaction (Table 6). The majority of the IIk-2 bacteria exhibited positive reactions for glucose, sucrose, and amygdalin oxidation while most of the IIk-1 strains were negative. All of the IIk strains showed variable reactivity in the remaining tests and the IIk-2 strains, for the most part, were more reactive than the IIk-1 organisms. Although tests for oxidase, dextrose oxidation and fermentation, nitrate reduction, growth on MacConkey agar, and motility are needed for the identification of the nonfermentative rods in addition to the 20 tests on the API strip, it is necessary to employ conventional media to obtain these results. The reactions for these tests were given in Tables 1-4. Due to the high degree of variability in the biochemical characteristics when using the API method, identifications were based on overall patterns rather than any single positive or negative result. The ATCC control cultures gave the expected patterns as shown in Table 8 and could be differentiated from the IIk strains.

B. ANTIBIOTIC SUSCEPTIBILITY PATTERNS

The results of the Kirby-Bauer susceptibility studies are summarized in Tables 9, 10, and 11. The IIk-1 organisms exhibited a higher degree of sensitivity to antibiotics than the strains of IIk-2. All 17 IIk-1 strains were sensitive to tetracycline, carbenicillin, and

TABLE 8

Biochemical Reactions of ATCC Control Cultures

Using the API Identification System

Tests or Substrates	*P. aeruginosa* ATCC 10145	*P. fluorescens* ATCC 13525	*P. cepacia* ATCC 10856	*P. maltophilia* ATCC 13637	*F. meningosepticum* ATCC 13253	*A. calcoaceticus* ATCC 23055	*X. campestris* ATCC 6402
ONPG	−	−	+	−	+	−	+
V-P	−	−	−	−	−	−	−
Glucose	+	−	+	−	−	−	−
Sucrose	−	−	+	−	−	−	−
Amygdalin	−	−	+	−	−	−	−
Citrate	+	+	+	+	+	+	+
Urease	+	−	−	−	−	−	−
Gelatin	+	−	−	+	+	−	+
Melibiose	−	−	−	−	−	−	−
Arabinose	+	−	−	−	−	−	−
TDA	−	−	−	−	−	−	−
Arginine dihydrolase	+	+	−	−	−	−	−
Lysine decarboxylase	−	−	+	+	−	−	−
Ornithine decarboxylase	−	−	−	−	−	−	−
H_2S	−	−	−	−	−	−	−
Indole	−	−	−	−	+	−	−
Mannitol	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−
Sorbitol	−	−	−	−	−	−	−
Rhamnose	−	−	−	−	−	−	−

TABLE 9a

Kirby-Bauer Susceptibility Test Results for

41 Strains of Group IIk

Organisms	Antibiotics													
	Tetracycline (Te) (30 mcg/ml)	Chloramphenicol (C) (30 mcg/ml)	Carbenicillin (Cb) (100 mcg/ml)	Erythromycin (E) (15 mcg/ml)	Vancomycin (Va) (30 mcg/ml)	Gentamicin (Gm) (10mcg/ml)	Kanamycin (K) (30 mcg/ml)	Tobramycin (NN) (10 mcg/ml)	Ampicillin (Am) (10 mcg/ml)	Clindamycin (Cm) (2 mcg/ml)	Penicillin (P) (10 units/ml)	Colistin (cs) (10 mcg/ml)	Cephalothin (Cl) (30 mcg/ml)	Oxacillin (So) (1 mcg/ml)
76-950 IIk-2	S[a]	I[b]	S	I	S	R[c]	R	R	R	R	R	R	R	R
76-940 IIk-2	S	I	S	I	I	R	R	R	R	R	R	R	R	R
76-944 IIk-2	S	I	I	I	R	R	R	R	R	R	R	R	R	R
74-1543 IIk-2	S	S	I	I	R	R	R	R	R	I	R	R	R	R
76-947 IIk-2	S	S	S	R	R	R	R	R	R	R	R	R	R	R
76-943 IIk-2	I	I	S	R	R	R	R	R	R	R	R	R	R	R
C 486 IIk-2	S	R	S	I	R	R	R	R	R	R	R	R	R	R
2147G IIk-2	R	R	I	R	R	R	R	R	R	R	R	R	R	R

TABLE 9b (cont'd)

Organisms	Antibiotics													
	Te	C	Cb	E	Va	Gm	K	NN	Am	Cm	P	Cs	Cl	So
2148G IIk-2	R	I	I	R	R	R	R	R	R	R	R	R	R	R
C 1822 IIk-2	S	S	R	S	R	R	R	R	R	S	R	R	R	R
C 198 IIk-2	S	S	I	S	S	R	R	R	R	R	R	R	R	R
76-945 IIk-2	S	S	R	I	S	R	R	R	R	R	R	R	R	R
LMC 3 IIk-2	S	R	S	S	S	R	R	R	R	R	R	R	R	R
2146G IIk-2	R	I	S	R	R	R	R	R	R	R	R	R	R	R
74-1540 IIk-2	S	S	S	I	R	S	S	R	R	R	R	R	R	R
76-946 IIk-2	S	S	R	S	S	R	R	R	R	S	R	R	R	R
74-1545 IIk-2	S	S	R	S	S	R	R	R	R	S	R	R	R	R
C 499 IIk-2	R	R	S	R	I	R	R	R	R	R	R	R	R	R
76-951 IIk-2	R	R	I	R	I	R	R	R	R	R	R	R	R	R
B 9042 IIk-2	S	S	S	S	R	R	R	R	R	S	R	R	R	R
76-952 IIk-2	S	S	I	S	R	R	R	R	R	S	R	R	R	R

TABLE 9c (cont'd)

Organisms	Antibiotics													
	Te	C	Cb	E	Va	Gm	K	NN	Am	Cm	P	Cs	Cl	So
B 8684 IIk-2	S	S	R	I	R	R	R	R	R	R	R	R	R	R
C 1279 IIk-2	S	S	R	I	R	R	R	R	R	R	R	R	R	R
76-949 IIk-2	S	S	R	I	R	R	R	R	R	R	R	R	R	R
1614G IIk-1	S	S	S	S	S	S	S	S	I	R	R	R	R	R
2123G IIk-1	S	S	S	S	S	S	S	S	S	R	I	R	R	R
76-934 IIk-1	S	S	S	S	S	S	S	S	R	R	R	R	R	R
76-941 IIk-1	S	S	S	S	R	R	R	R	R	R	R	R	R	R
1672G IIk-1	S	S	S	S	S	S	S	S	S	R	R	R	R	R
LMC 4 IIk-1	S	S	S	S	S	S	S	S	S	R	S	R	R	R
76-933 IIk-1	S	S	S	S	S	S	S	S	S	R	R	R	R	R
76-942 IIk-1	S	S	S	S	S	S	R	S	S	R	I	R	R	R
K 2 IIk-1	S	S	S	S	S	S	S	S	S	R	S	R	S	R
LMC 1 IIk-1	S	S	S	S	S	S	S	S	S	R	R	R	R	R

TABLE 9d (cont'd)

Organisms	Antibiotics													
	Te	C	Cb	E	Va	Gm	K	NN	Am	Cm	P	Cs	Cl	So
LMC 2 IIk-1	S	S	S	S	S	S	S	S	S	R	R	R	R	R
1897G IIk-1	S	S	S	S	S	S	S	S	R	R	R	R	R	R
C 1196 IIk-1	S	S	S	S	S	S	S	S	R	R	R	R	R	R
1852G IIk-1	S	S	S	S	S	S	S	S	S	R	R	R	R	R
1854G IIk-1	S	S	S	S	I	S	S	S	I	R	R	R	R	R
2232G IIk-1	S	S	S	S	S	S	S	S	S	R	R	S	R	R
K 1 IIk-1	S	I	S	S	S	S	S	S	S	R	R	S	R	R

[a]S = Sensitive
[b]I = Indeterminate
[c]R = Resistant

TABLE 10

Kirby-Bauer Susceptibility Test Results for ATCC Control Organisms

Organisms	Antibiotics													
	Tetracycline (30 mcg/ml)	Chloramphenicol (30 mcg/ml)	Carbenicillin (100 mcg/ml)	Erythromycin (15 mcg/ml)	Vancomycin (30 mcg/ml)	Gentamicin (10 mcg/ml)	Kanamycin (30 mcg/ml)	Tobramycin (10 mcg/ml)	Ampicillin (10 mcg/ml)	Clindamycin (2 mcg/ml)	Penicillin (10 units/ml)	Colistin (10 mcg/ml)	Cephalothin (30 mcg/ml)	Oxacillin (1 mcg/ml)
<u>A. calcoaceticus</u> ATCC 23055	S	S	S	S	S	S	S	S	S	R	S	S	S	S
<u>X. campestris</u> ATCC 6402	S	R	R	S	S	S	S	S	R	R	R	R	R	R
<u>P. maltophilia</u> ATCC 13637	R	S	S	R	R	S	S	R	R	R	R	R	R	R
<u>P. fluorescens</u> ATCC 13525	S	R	R	R	R	S	S	S	R	R	R	R	R	R
<u>P. aeruginosa</u> ATCC 10145	R	R	I	R	R	R	R	S	R	R	R	S	R	R
<u>F. meningosepticum</u> ATCC 13253	R	R	R	R	S	R	R	R	R	R	R	R	R	R
<u>P. cepacia</u> ATCC 10856	R	I	R	R	R	R	R	R	R	R	R	R	R	R

TABLE 11

Antimicrobial Susceptibility Patterns of Groups IIk-1 and IIk-2

Antibiotics	Group IIk-1				Group IIk-2			
	Number Sensitive	Number Resistant	Number Indeterminate	Percent Sensitive	Number Sensitive	Number Resistant	Number Indeterminate	Percent Sensitive
Tetracycline	17	0	0	100	18	5	1	75
Carbenicillin	17	0	0	100	10	7	7	42
Erythromycin	17	0	0	100	7	7	10	29
Chloramphenicol	16	0	1	94	13	5	6	54
Gentamicin	16	1	0	94	1	23	0	4
Tobramycin	16	1	0	94	0	24	0	0
Kanamycin	15	2	0	88	1	23	0	4
Vancomycin	15	1	1	88	6	15	3	25
Ampicillin	11	4	2	65	0	24	0	0
Penicillin	2	13	2	12	0	24	0	0
Colistin	2	15	0	12	0	24	0	0
Cephalothin	1	16	0	6	0	24	0	0
Clindamycin	0	17	0	0	5	18	1	21
Oxacillin	0	17	0	0	0	24	0	0

erythromycin (Table 11). Most strains were also susceptible to chloramphenicol, gentamicin, kanamycin, vancomycin, tobramycin, and ampicillin. All of the IIk-1 bacteria were resistant to clindamycin and oxacillin. The majority were also resistant to penicillin, colistin, and cephalothin.

In contrast, the IIk-2 organisms were not uniformly susceptible to any one antibiotic (Table 11). Only 75% (18/24) of the IIk-2 strains were sensitive to tetracycline. A large number of indeterminate results were obtained when the IIk-2 strains were tested against chloramphenicol, carbenicillin, and erythromycin. All of the IIk-2 organisms were resistant to tobramycin, ampicillin, penicillin, cephalothin, oxacillin, and colistin. Most were resistant to clindamycin, gentamicin, and kanamycin.

The ATCC control organisms exhibited the expected susceptibility patterns with _P. aeruginosa_ ATCC 10145, _P. cepacia_ ATCC 10856, and _F. meningosepticum_ ATCC 13253 being highly resistant (Table 10). _A. calcoaceticus_ ATCC 23055 showed the highest degree of sensitivity to the antibiotics tested.

C. TUBE AGGLUTINATION STUDIES

Antisera were prepared against 14 strains of both the IIk-1 and IIk-2 biotypes as well as 5 of the 7 control ATCC strains. Each of the 33 antisera were reacted against each of the IIk-1, IIk-2, and control ATCC strains in tube

agglutination assays. The titers of the anti-IIk-1, anti-IIk-2 and anti-ATCC control antisera that reacted with the test organisms are shown in Tables 12, 13, and 14, respectively. All of the organisms were readily agglutinated by the homologous antiserum. Homologous titers ranged from 40 to 2560. Titers obtained in reciprocal tests for cross-reactivity were generally lower; however, a few strains were agglutinated in heterologous antisera at the same or even higher titers than in the homologous antiserum (e.g. IIk-1 strain 76-934 in anti-1614G and anti-2123G). None of the organisms agglutinated in normal rabbit serum.

The results indicated that the IIk strains vary in their antigenic makeup, since no one antiserum agglutinated all strains of a particular biotype. Very little cross-reactivity was evident between the IIk-1 and IIk-2 biotypes. Patterns of reactivity were noted within a particular biotype which suggested the existence of subgroups (e.g. a IIk-1 subgroup consisting of organisms 1614G, 2123G, 76-934, and 76-941 and a IIk-2 subgroup consisting of organisms 76-950, 76-940, 76-944, 74-1543, 76-947, 76-943, and C 486).

Although very little cross-reactivity was observed between the IIk organisms and the anti-ATCC control antisera, two such reactions did occur. Strain 76-945 IIk-2 produced a titer of 1:20 when tested with anti-*F. meningosepticum* ATCC 13253 and 1852G IIk-1 exhibited a titer of 1:160 when reacted with anti-*P. fluorescens* ATCC 13525. None of the

TABLE 12a

Agglutination Reactions of 14 Anti-IIk-1

Antisera and Cross-Reacting Organisms

Organisms[a]	Antisera													
IIk-1	1614G	2123G	76-934	1672G	LMC 4	K 2	LMC 1	LMC 2	1897G	C 1196	1852G	1854G	2232G	K 1
1614G	80[b]	40	40	-[c]	80	-	-	-	-	-	40	40	-	-
2123G	80	160	40	-	-	-	-	-	-	-	-	-	-	-
76-934	640	640	160	-	-	-	-	-	-	-	-	-	-	-
76-941	40	40	20	-	-	-	-	-	-	-	-	-	-	-
1672G	-	-	-	80	160	20	-	-	-	-	-	-	-	-
LMC 4	-	20	-	80	320	40	-	-	-	-	-	-	-	-
76-942	-	-	-	-	20	80	-	-	-	-	-	-	-	-
76-933	-	40	-	20	40	80	-	-	-	-	-	20	-	-
K 2	-	-	40	-	-	320	-	-	40	-	80	40	-	-
LMC 1	-	-	-	-	-	20	40	20	20	-	-	-	-	-

TABLE 12b (cont'd)

Organisms[a]	Antisera													
	1614G	2123G	76-934	1672G	LMC 4	K 2	LMC 1	LMC 2	1897G	C 1196	1852G	1854G	2232G	K 1
IIk-1														
LMC 2	-	20	20	-	40	-	40	320	40	20	40	-	-	-
1897G	-	-	-	-	-	-	160	80	80	-	-	-	-	-
C 1196	-	20	-	-	-	-	-	-	-	160	-	-	-	-
1852G	-	-	-	-	-	-	-	-	-	-	160	-	-	-
1854G	-	-	-	-	-	-	-	-	-	-	-	40	-	-
K 1	-	-	-	-	-	-	-	-	-	-	-	-	20	80
IIk-2														
B 8684	40	-	-	-	-	-	-	-	-	-	-	-	-	-
C 1279	20	-	-	-	-	-	-	-	-	-	-	-	-	-
76-949	20	-	-	-	-	-	-	-	-	-	-	-	-	-
P. fluorescens ATCC 13525	-	-	-	-	-	-	-	-	-	-	160	-	-	-

[a]All organisms were negative for 2+ agglutination in a 1:20 dilution of normal rabbit serum.
[b]Highest serum dilution resulting in at least a 2+ agglutination.
[c]Absence of 2+ agglutination in lowest serum dilution tested (1:20).

TABLE 13a

Agglutination Reactions of 14 Anti-IIk-2 Antisera and Cross-Reacting Organisms

Organisms[a]	Antisera													
IIk-2	74-1543	76-947	C 486	2147G	2148G	C 1822	C 198	2146G	LMC 3	76-946	C 499	B 9042	B 8684	C 1279
76-950	640[b]	80	160	–[c]	–	–	40	–	–	–	–	–	–	–
76-940	640	80	160	–	–	–	–	–	–	–	–	–	–	–
76-944	320	80	160	–	–	–	–	–	20	–	–	–	–	–
74-1543	640	80	160	–	–	–	–	–	–	–	–	–	–	–
76-947	320	160	80	–	–	–	–	–	–	–	–	–	–	–
76-943	320	80	80	–	–	–	–	–	–	–	–	–	–	–
C 486	640	80	160	–	–	–	–	–	–	–	–	–	–	–
2147G	40	–	80	160	640	80	80	40	160	20	80	40	40	80
2148G	–	–	–	320	320	–	–	–	20	–	–	–	20	–

TABLE 13b (cont'd)

Organisms[a]	Antisera													
IIk-2	74-1543	76-947	C 486	2147G	2148G	C 1822	C 198	2146G	LMC 3	76-946	C 499	B 9042	B 8684	C 1279
C 1822	-	-	-	20	40	640	20	-	40	20	-	-	-	20
C 198	-	-	-	-	-	-	80	-	-	-	-	-	-	-
76-945	-	-	-	20	-	40	-	20	-	-	-	-	20	20
2146G	-	-	-	-	-	-	-	320	-	-	-	-	-	-
LMC 3	20	-	-	40	40	-	20	80	1280	-	-	-	-	-
74-1540	-	-	-	-	-	-	-	40	-	-	-	-	-	-
76-946	-	-	-	-	-	-	-	-	-	640	-	-	-	-
74-1545	-	-	-	-	-	-	-	-	-	640	-	-	-	-
C 499	-	-	-	-	-	-	-	-	-	-	80	-	-	-
76-951	-	-	-	20	-	-	-	-	-	-	160	-	-	-
B 9042	-	-	-	-	-	-	-	-	-	-	-	320	-	-
76-952	-	-	-	-	-	-	-	-	-	-	-	160	-	-

TABLE 13c (cont'd)

Organisms[a]	Antisera													
	75-1543	76-947	C 486	2147G	2148G	C 1822	C 198	2146G	LMC 3	76-946	C 499	B 9042	B 8684	C 1279
IIk-2														
B 8684	-	-	-	-	-	-	-	-	-	-	-	-	<u>160</u>	160
C 1279	-	-	-	-	-	-	-	-	-	-	-	-	160	<u>160</u>
76-949	-	-	-	-	-	-	-	-	-	-	-	-	80	40
LMC 2 IIk-1	-	-	20	-	-	-	-	-	-	-	20	-	-	-
P. cepacia ATCC 10856	-	-	-	-	-	-	160	-	-	-	-	-	-	-
X. campestris ATCC 6402	-	-	-	-	-	-	320	-	-	-	-	-	-	-
F. meningosepticum ATCC 13253	-	-	-	-	-	40	-	-	-	-	-	-	-	-
A. calcoaceticus ATCC 23055	-	-	-	-	-	-	20	-	-	-	-	-	-	-

[a]All organisms were negative for 2+ agglutination in a 1:20 dilution of normal rabbit serum.
[b]Highest serum dilution resulting in at least a 2+ agglutination.
[c]Absence of 2+ agglutination in lowest serum dilution tested (1:20).

TABLE 14

Agglutination Reactions of 5 Anti-ATCC Control Antisera and Cross-Reacting Organisms

Organisms[a]	Antisera				
	P. fluorescens ATCC 13525	P. cepacia ATCC 10856	X. campestris ATCC 6402	F. meningosepticum ATCC 13253	A. calcoaceticus ATCC 23055
P. fluorescens ATCC 13525	640[b]	-[c]	-	-	-
P. cepacia ATCC 10856	-	2560	80	-	-
X. campestris ATCC 6402	-	640	320	-	-
F. meningosepticum ATCC 13253	-	-	-	1280	-
A. calcoaceticus ATCC 23055	-	-	-	-	80
76-945 IIk-2	-	-	-	20	-
1852G IIk-1	160	-	-	-	-

[a]All organisms were negative for 2+ agglutination in a 1:20 dilution of normal rabbit serum.

[b]Highest serum dilution resulting in at least a 2+ agglutination.

[c]Absence of 2+ agglutination in lowest serum dilution tested (1:20).

other IIk organisms reacted with any of the anti-ATCC control antisera.

The anti-IIk antisera were also relatively inactive when tested with the ATCC organisms. However, anti-C 1822 IIk-2 exhibited a titer of 1:40 versus *F. meningosepticum* ATCC 13253, anti-1852G IIk-1 showed a 1:160 titer against *P. fluorescens* ATCC 13525, and anti-C 198 IIk-2 was slightly more reactive exhibiting a titer of 1:160 versus *P. cepacia* ATCC 10856, 1:320 versus *X. campestris* ATCC 6402, and 1:20 versus *A. calcoaceticus* ATCC 23055.

D. INDIRECT FLUORESCENT ANTIBODY STAINS

A panel of 8 anti-IIk-2 and 6 anti-IIk-1 antisera was selected for use in indirect fluorescent antibody studies. The results are summarized in Table 15. Thirteen IIk-2 strains were screened with a 1:10 dilution of each antiserum and a number of the positives were titered. The reaction patterns obtained from these experiments were similar to those obtained in the tube agglutination tests. However, the indirect fluorescent technique yielded a greater number of positives. Reactivity of the IIk-1 antisera with the IIk-2 organisms still remained minimal, but a number of the IIk-2 organisms were more reactive with the IIk-2 antisera. Organisms 2147G, C 1822, 2148G, and 76-946 reacted with all IIk-2 antisera, yet no one antiserum reacted with all of the IIk-2 strains tested.

TABLE 15a

Indirect Fluorescent Antibody Stain Results on 13 Strains of Group IIk-2

Organisms[a]	Anti-IIk-2 Antisera								IIk-1 Antisera					
IIk-2	74-1543	2147G	C 198	2146G	76-946	C 499	B 9042	B 8684	76-934	K 2	LMC 1	C 1196	1854G	2232G
76-947	2560[b]	–[c]	–	–	–	–	–	–	–	–	–	≥10	–	–
74-1543	640	–	–	–	–	–	–	–	–	–	–	–	–	–
2147G	80	320	160	80	160	40	40	160	≥10	–	–	–	–	–
C 1822	≥10	≥10	40	≥10	40	≥10	≥10	≥10	–	–	–	–	–	–
LMC 3	10	80	40	20	–	–	≥10	≥10	–	–	–	–	–	–
76-945	–	20	40	20	–	–	–	–	–	–	–	–	–	–
2148G	≥10	320	≥10	≥10	≥10	≥10	≥10	80	–	–	–	–	–	–
C 198	–	–	160	–	–	–	–	–	–	–	–	–	–	–
2146G	–	–	–	320	–	–	–	–	–	–	–	–	–	–
74-1540	–	≥10	–	40	≥10	–	–	≥10	–	–	–	–	–	–
74-1545	–	–	–	–	320	≥10	≥10	≥10	–	–	–	–	–	–

TABLE 15 b (cont'd)

Organisms[a]	Anti-IIk-2 Antisera								IIk-1 Antisera					
	74-1543	2147G	C 198	2146G	76-946	C 499	B 9042	B 8684	76-934	K 2	LMC 1	C 1196	1854G	2232G
IIk-2														
76-946	≥10	≥10	≥10	≥10	640	≥10	≥10	≥10	-	-	-	-	-	-
76-951	-	10	-	-	≥10	80	≥10	-	-	-	-	-	-	-
C 499	ND[d]	ND	ND	ND	ND	160	ND	ND	ND	ND	ND	ND	ND	ND
B 9042	ND	ND	ND	ND	ND	ND	1280	ND	ND	ND	ND	ND	ND	ND
B 8684	ND	ND	ND	ND	ND	ND	ND	320	ND	ND	ND	ND	ND	ND
IIk-1														
76-934	ND	ND	ND	ND	ND	ND	ND	ND	≥10	ND	ND	ND	ND	ND
K 2	ND	ND	ND	ND	ND	ND	ND	ND	ND	≥10	ND	ND	ND	ND
LMC 1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	≥10	ND	ND	ND
C 1196	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	≥10	ND	ND
1854G	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	≥10	ND
2232G	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	≥10

[a]All organisms were negative for 2+ fluorescence in a 1:10 dilution of normal rabbit serum.
[b]Highest serum dilution resulting in at least a 2+ fluorescence.
[c]Absence of 2+ fluorescence at lowest serum dilution tested (1:10).
[d]Not done.

E. ADSORPTION STUDIES

A panel of 17 antisera was chosen which would, hopefully, detect all of the IIk organisms. The panel included: anti-76-934, anti-LMC 4, anti-K 2, anti-LMC 1, anti-C 1196, anti-1852G, anti-1854G, anti-2232G, anti-74-1543, anti-2147G, anti-C 1822, anti-C 198, anti-2146G, anti-76-946, anti-C 499, anti-B 9042, and anti-B 8684. Each of the antisera was adsorbed with each of the apparent cross-reacting organisms and then the remaining titers against the organisms which had reacted with the unadsorhed antiserum were redetermined. The results of these studies are included in Tables 16 through 32.

In the initial experiments, it was discovered that adsorption of the IIk-1 antisera with IIk-1 strain K 2 resulted in an increase in some titers (Tables 16, 19, 20, 21, and 23) and in the complete adsorption of others (Tables 16-23). Strain K 2 also reacted with a number of anti-IIk-1 antisera, suggesting the possibility that the agglutinating antibodies were directed against "core" antigens (12). Therefore, in the remaining experiments, each antiserum in the anti-IIk-1 panel was adsorbed first with organism K 2 to determine the "true" titer, and then the antiserum was adsorbed with each of the remaining, apparent cross-reactive bacteria. Adsorption with IIk-1 strain K 2 removed the reactivity of: anti-76-934 against IIk-1 strains K 2 and LMC 2 (Table 16); anti-LMC 4 against IIk-1 strains 1614G,

TABLE 16

Agglutination Reactions of IIk Strains with

Unadsorbed and Adsorbed Anti-76-934

Organisms	Unadsorbed	Adsorbed with						
		K 2	LMC 2	K 2 and 1614G	K 2 and 2123G	K 2 and 76-934	K 2 and 76-941	76-934
1614G IIk-1	40[a]	160	160	<20[b]	<20	<20	<20	<20
2123G IIk-1	40	640	640	<20	<20	<20	<20	<20
76-934 IIk-1	160	320	320	<20	<20	<20	<20	<20
76-941 IIk-1	20	160	160	<20	<20	<20	<20	<20
K 2 IIk-1	40	<20	<20	<20	<20	<20	<20	<20
LMC 2 IIk-1	20	<20	<20	<20	<20	<20	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 17

Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-LMC 4

Organisms	Unadsorbed	Adsorbed with			
		K 2	K 2 and 1672G	K 2 and LMC 4	LMC 4
1672G IIk-1	160[a]	320	<20[b]	<20	<20
LMC 4 IIk-1	320	320	<20	<20	<20
1614G IIk-1	80	<20	<20	<20	<20
76-942 IIk-1	20	<20	<20	<20	<20
76-933 IIk-1	40	<20	<20	<20	<20
LMC 2 IIk-1	40	<20	<20	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 18

Agglutination Reactions of IIk Strains with

Unadsorbed and Adsorbed Anti-K 2

Organisms	Unadsorbed	Adsorbed with					
		1672G	LMC 4	76-942	76-933	K 2	LMC 1
1672G IIk-1	20[a]	<20[b]	<20	<20	<20	<20	<20
LMC 4 IIk-1	40	<20	<20	<20	<20	<20	<20
76-942 IIk-1	80	<20	<20	<20	<20	<20	<20
76-933 IIk-1	80	<20	<20	<20	<20	<20	<20
K 2 IIk-1	320	<20	<20	<20	<20	<20	<20
LMC 1 IIk-1	20	<20	<20	<20	<20	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 19

Agglutination Reactions of IIk Strains with

Unadsorbed and Adsorbed Anti-LMC 1

Organisms	Unadsorbed	Adsorbed with				
		K 2	K 2 and LMC 1	K 2 and LMC 2	K 2 and 1897G	LMC 1
LMC 1 IIk-1	40[a]	320	<20[b]	<20	<20	<20
LMC 2 IIk-1	40	320	<20	<20	<20	<20
1897G IIk-1	160	160	<20	<20	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 20

Agglutination Reactions of IIk Strains with

Unadsorbed and Adsorbed Anti-C 1196

Organisms	Unadsorbed	Adsorbed with			
		K 2	K 2 and 76-947	K 2 and C 1196	C 1196
76-947 IIk-2	80[a]	40	<20[b]	<20	<20
C 1196 IIk-1	160	320	<20	<20	<20
LMC 2 IIk-1	20	<20	<20	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 21

Agglutination Reactions of IIk and Control Strains with Unadsorbed and Adsorbed Anti-1852G

Organisms	Unadsorbed	Adsorbed with			
		K 2	K 2 and 1852G	K 2 and *P. fluorescens* ATCC 13525	1852G
1614G IIk-1	40[a]	<20[b]	<20	<20	<20
K 2 IIk-1	80	<20	<20	<20	<20
LMC 2 IIk-1	40	<20	<20	<20	<20
1852G IIk-1	160	320	<20	160	<20
P. fluorescens ATCC 13525	160	640	<20	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 22

Agglutination Reactions of IIk Strains with Unadsorbed and Adsorbed Anti-1854G

Organisms	Unadsorbed	Adsorbed with		
		K 2	K 2 and 1854G	1854G
1614G IIk-1	40[a]	<20[b]	<20	<20
76-933 IIk-1	20	<20	<20	<20
K 2 IIk-1	40	<20	<20	<20
1854G IIk-1	40	320	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 23

Agglutination Reactions of IIk Strains with

Unadsorbed and Adsorbed Anti-2232G

Organisms	Unadsorbed	Adsorbed with			
		K 2	K 2 and 2232G	K 2 and K 1	2232G
2232G IIk-1	160[a]	80	<20[b]	<20	<20
K 1 IIk-1	20	320	<20	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 24

Agglutination Reactions of IIk Strains with

Unadsorbed and Adsorbed Anti-74-1543

Organisms	Unad-sorbed	Adsorbed with									
		76-950	76-940	76-944	74-1543	76-947	76-943	C 486	2147G	LMC 3	LMC 2
76-950 IIk-2	640[a]	<20[b]	<20	<20	<20	<20	<20	<20	640	1280	640
76-940 IIk-2	640	<20	<20	<20	<20	<20	<20	<20	640	1280	640
76-944 IIk-2	320	<20	<20	<20	<20	<20	<20	<20	640	640	640
74-1543 IIk-2	640	<20	<20	<20	<20	<20	<20	<20	640	640	640
76-947 IIk-2	320	<20	<20	<20	<20	<20	<20	<20	640	640	320
76-943 IIk-2	640	<20	<20	<20	<20	<20	<20	<20	640	640	640
C 486 IIk-2	640	<20	<20	<20	<20	<20	<20	<20	1280	320	640
2147G IIk-2	40	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
LMC 3 IIk-2	20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
LMC 2 IIk-1	20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 25

Agglutination Reactions of IIk Strains with

Unadsorbed and Adsorbed Anti-2147G

Organisms	Unadsorbed	Adsorbed with					
		2147G	2148G	C 1822	76-945	LMC 3	76-951
2147G IIk-2	160[a]	<20[b]	<20	320	320	640	320
2148G IIk-2	320	<20	<20	160	160	160	160
C 1822 IIk-2	20	<20	<20	<20	<20	<20	<20
76-945 IIk-2	20	<20	<20	<20	<20	<20	<20
LMC 3 IIk-2	40	<20	<20	<20	<20	<20	<20
76-951 IIk-2	20	<20	<20	<20	<20	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 26

Agglutination Reactions of IIk and Control Strains

With Unadsorbed and Adsorbed Anti-C 1822

Organisms	Unadsorbed	Adsorbed with			
		2147G	76-945	*F. meningosepticum* ATCC 13253	C 1822
2147G IIk-2	80[a]	<20[b]	<20	<20	<20
76-945 IIk-2	40	<20	<20	<20	<20
F. meningosepticum ATCC 13253	40	40	80	<20	<20
C 1822 IIk-2	640	320	640	320	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 27

Agglutination Reactions of IIk and Control Strains with Unadsorbed and Adsorbed Anti-C 198

Organisms	Unadsorbed	Adsorbed with							
		76-950	2147G	C 1822	LMC 3	C 198	*P. cepacia* ATCC 10856	*X. campestris* ATCC 6402	*A. calcoaceticus* ATCC 23055
76-950 IIk-2	40[a]	<20[b]	<20	<20	<20	<20	<20	<20	20
2147G IIk-2	80	<20	<20	<20	<20	<20	20	20	40
C 1822 IIk-2	20	<20	<20	<20	<20	<20	<20	<20	<20
LMC 3 IIk-2	20	<20	<20	<20	<20	<20	<20	<20	<20
C 198 IIk-2	80	320	640	320	320	<20	160	320	160
P. cepacia ATCC 10856	160	160	160	320	320	<20	<20	40	320
X. campestris ATCC 6402	320	160	160	320	160	<20	<20	<20	160
A. calcoaceticus ATCC 23055	20	<20	<20	<20	<20	<20	<20	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 28

Agglutination Reactions of IIk Strains with

Unadsorbed and Adsorbed Anti-2146G

Organisms	Unadsorbed	Adsorbed with				
		2147G	76-945	74-1540	LMC 3	2146G
2147G IIk-2	40[a]	<20[b]	<20	<20	<20	<20
76-945 IIk-2	20	<20	<20	<20	<20	<20
74-1540 IIk-2	40	<20	<20	<20	<20	<20
LMC 3 IIk-2	80	80	80	80	<20	<20
2146G IIk-2	320	640	640	320	640	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 29

Agglutination Reactions of IIk Strains with

Unadsorbed and Adsorbed Anti-76-946

Organisms	Unadsorbed	Adsorbed with			
		2147G	C 1822	76-946	74-1545
2147G IIk-2	20[a]	<20[b]	<20	<20	<20
C 1822 IIk-2	20	<20	<20	<20	<20
76-946 IIk-2	640	320	320	<20	<20
74-1545 IIk-2	640	320	320	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 30

Agglutination Reactions of IIk Strains with

Unadsorbed and Adsorbed Anti-C 499

Organisms	Unadsorbed	Adsorbed with			
		2147G	LMC 2	C 499	76-951
2147G IIk-2	80[a]	<20[b]	<20	<20	<20
LMC 2 IIk-1	20	<20	<20	<20	<20
C 499 IIk-2	80	80	80	<20	<20
76-951 IIk-2	160	160	160	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 31

Agglutination Reactions of IIk Strains with

Unadsorbed and Adsorbed Anti-B 9042

Organisms	Unadsorbed	Adsorbed with		
		2147G	B 9042	76-952
2147G IIk-2	40[a]	<20[b]	<20	<20
B 9042 IIk-2	160	160	<20	<20
76-952 IIk-2	320	160	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

TABLE 32

<u>Agglutination Reactions of IIk Strains with</u>

<u>Unadsorbed and Adsorbed Anti-B 8684</u>

Organisms	Unadsorbed	Adsorbed with					
		2147G	2148G	76-945	B 8684	C 1279	76-949
2147G IIk-2	40[a]	<20[b]	<20	<20	<20	<20	<20
2148G IIk-2	20	<20	<20	<20	<20	<20	<20
76-945 IIk-2	20	<20	<20	<20	<20	<20	<20
B 8684 IIk-2	160	160	160	160	<20	<20	<20
C 1279 IIk-2	160	160	160	320	<20	<20	<20
76-949 IIk-2	80	80	160	160	<20	<20	<20

[a]Highest serum dilution resulting in at least a 2+ agglutination.

[b]Absence of 2+ agglutination at a 1:20 serum dilution.

76-942, 76-933, and LMC 2 (Table 17); anti-C 1196 against IIk-1 strain LMC 2 (Table 20); anti-1852G with IIk-1 strains 1614G, K 2, and LMC 2 (Table 21); and of anti-1854G with IIk-1 strains 1614G, 76-933, and K 2 (Table 22).

A somewhat similar phenomenon occurred in the anti-IIk-2 series of adsorptions with strain 2147G, which reacted with all the antisera in the anti-IIk-2 panel. Although adsorption of the anti-IIk-2 antisera with 2147G did not generally result in an increase in the homologous titer it did remove the apparent cross-reactivity of: anti-74-1543 against IIk-2 strains 2147G and LMC 3 and IIk-1 strain LMC 2 (Table 24); anti-2147G against IIk-2 strains C 1822, 76-945, LMC 3, and 76-951 (Table 25); anti-C 1822 against IIk-2 strains 2147G and 76-945 (Table 26); anti-C 198 against IIk-2 strains 76-950, 2147G, C 1822, and LMC 3 and ATCC strain 23055 (Table 27); anti-2146G against IIk-2 strains 2147G, 76-945, and 74-1540 (Table 28); anti-76-946 against IIk-2 strains 2147G and C 1822 (Table 29); anti-C 499 against IIk-2 strain 2147G and IIk-1 strain LMC 2 (Table 30); anti-B 9042 against IIk-2 strain 2147G (Table 31); and anti-B 8684 against IIk-2 strains 2147G, 2148G, and 76-945 (Table 32). In only one instance, adsorption of anti-C 198 with 2147G, was a rise in titer seen when the anti-IIk-2 antisera was adsorbed with the polyagglutinating strain and retested against the homologous organism.

In most instances, two adsorptions with any of the

remaining apparent cross-reactive organisms were sufficient to remove the titers which had been detected against the organisms with the K 2 and 2147G adsorbed antisera. A non-reciprocal pattern of cross-reactivity was observed in both series of adsorptions. For example, adsorption of anti-1852G with 1852G removed the reaction against _P. fluorescens_ ATCC 13525 but adsorption with _P. fluorescens_ ATCC 13525 did not remove the reaction against 1852G (Table 21); adsorption of anti-C 1822 with _F. meningosepticum_ ATCC 13253 did not remove the reaction of the antiserum against C 1822 but adsorption with C 1822 was able to eliminate the reaction against _F. meningosepticum_ ATCC 13253 (Table 26); adsorption of anti-C 198 with _P. cepacia_ ATCC 10856 and _X. campestris_ ATCC 6402 did not remove the reaction against C 198, but adsorption with C 198 removed the reactions against _P. cepacia_ ATCC 10856 and _X. campestris_ ATCC 6402 (Table 27); also, adsorption of anti-2146G with 2146G eliminated the reaction of the antiserum with 2146G and LMC 3, but adsorption with LMC 3 was not able to eliminate the reaction with 2146G.

The adsorption sutdies suggested that there are at least 8 IIk-1 subgroups represented by the following organisms: Subgroup I, including 1614G, 2123G, 76-934, and 76-941; Subgroup II, including 1672G and LMC 4; Subgroup III, including K 2, 76-933, and 76-942; Subgroup IV, including LMC 1, LMC 2, and 1897G; Subgroup V, including C 1196; Subgroup VI, including 1852G; Subgroup VII, including 1854G;

and Subgroup VIII, including 2232G and K 1.

There appears to be at least 9 subgroups of the IIk-2 biotypes represented by the following organisms: Subgroup I, including 76-950, 76-940, 76-944, 74-1543, 76-947, 76-943, and C 486; Subgroup II, including 2147G and 2148G; Subgroup III, including C 1822; Subgroup IV, including 2146G and possibly LMC 3; Subgroup V, including C 198, Subgroup VI, including 76-946 and 74-1545; Subgroup VII, including C 499; Subgroup VIII, including B 9042 and 76-952; and Subgroup IX, including B 8684, C 1279, and 76-949.

DISCUSSION

Over the past several years, our laboratory has noted an increase in the rate of isolation of the unusual nonfermentative gram negative rods, particularly strains of Group IIk, from blood and other clinical specimens. For example, no IIk organisms were isolated from blood cultures in 1972 but in 1977 IIk-1 was isolated 77 times. Von Graevenitz (40)and Grehn (personal communication from the University of Mannheim in Mannheim, Germany) have reported similar occurrences. Evaluation of the role of these organisms in infection, however, has been hampered by errors in identification due to the lack of availability of a simple and accurate method for their identification.

It has been suggested that the API 20 Enteric strip, which has been used successfully for the identification of the *Enterobacteriaceae*, might also prove useful to differentiate the nonfermentative gram negative rods (1). Thus, we decided to evaluate the applicability of the API 20 strip for the identification of the IIk organisms.

The results obtained from the API 20 strip were compared to those obtained with the conventional biochemical techniques which are presently used by most clinical laboratories (14, 22, 24, 38). Group IIk could be readily

distinguished from P. aeruginosa, P. fluorescens, P. maltophilia, F. meningosepticum, and A. calcoaceticus by the conventional system. Data previously published by Gilardi (22, 24) support these conclusions. The separation of Group IIk from strains of P. cepacia, however, did not prove to be as simple. P. cepacia is usually differentiated from Group IIk on the basis of positive tests for oxidation of mannitol, decarboxylation of lysine, and production of lipase. It was noted, however, that not all strains of P. cepacia are positive for these tests. Therefore, these biochemical criteria do not always suffice to classify these organisms or to adequately separate all of the nonfermenters from Group IIk.

Separation of Group IIk-1 from IIk-2 is also difficult by biochemical means. Dependence upon the production of urease and growth on MacConkey agar as suggested by Weaver et al. (42) did not prove to be practical. Of the 17 strains of IIk-1 bacteria studied, 4 out of 17 (24%) were positive for both of these characteristics while 92% (22/24) of the IIk-2 strains were positive for urease and only 88% (21/24) were positive for growth on MacConkey agar. The TSI reaction did aid in distinguishing most, but not all, strains of IIk-1 (KK--) from IIk-2 (AK--). The production of a bright yellow pigment at 25 C and 35 C by the IIk-1 strains, however, appeared to be the most consistent

differentiating characteristic. Five of the 24 IIk-2 strains produced a very light yellow pigment, but this usually appeared only after 48-72 h of incubation at room temperature. Dependence upon pigment production alone, however, would not appear to be a reliable method for differentiating these biotypes.

Moreover, the conventional system proved to be time consuming to use and required exacting technique. A very heavy inoculum had to be used in order to achieve consistent reactions with most of the tests. Many of the nonfermenters, including IIk-1, grow slowly and require 48 h before enough growth is obtained to inoculate the biochemical tests. It was also necessary, at times, to incubate the biochemicals for 2-7 days for reactions to occur. Reading the reactions of the OF carbohydrates was very difficult. For example, oxidation of a sugar is indicated by a color change from blue-green to yellow beginning at the top of the medium and moving down. Weak oxidizers may produce only a pale green or yellow-green reaction, so that a weak positive may go unobserved. Yellow pigmented organisms can also mislead the investigator and result in the reporting of false positives. Therefore, it is necessary to employ an inoculated tube of OF basal medium without added carbohydrate as a negative control. Even with proper controls, interpretation still remains subjective.

The API 20 proved to be less cumbersome; since only one or two colonies were required to inoculate the 20 tests. Identifications were often made after 24 h of incubation, although 48 h of incubation was sometimes required for the slow growing organisms. The Group IIk strains were a particular problem in this regard and, most often, required the full 48 h before the strips could be read.

The results of the API 20 E system were in agreement with those obtained by the conventional method for 93% (38/41) of the IIk strains tested. Discrepancies were noted with organisms 76-1545, K 2, and 2146G. Organism 74-1545, which was obtained from API, was identified as a IIk-1 by the Weaver method, while the API strip identified it as a IIk-2. Tube agglutination studies, which will be discussed in detail later, confirmed the identification of this strain as a IIk-2 (Table 13b). Strain K 2 had a positive TDA, while all IIk bacteria in the API register have been reported as negative. The API computer service indicated that the organism most closely resembles IIk-2, since none of the IIk-1 organisms in the API Profile Register are arabinose positive. In contrast, K 2 was identified as a "typical" IIk-1 by the Weaver method and this was later confirmed by the serological studies (Table 12a). Strain 2146G could not be readily identified by either of the identification systems. The organism grew well on MacConkey agar, was weakly urease positive and had a KK--

TSI reaction. On the basis of these reactions, the organism was identified as a IIk-2 biotype in the conventional scheme. According to the API computer service, the organism most closely resembles *P. cepacia*, since it is gelatinase positive, while all IIk-2 strains are negative. Tube agglutination studies indicated that this organism is a IIk-2 biotype (Table 13b).

Six of the 7 control organisms were identified correctly using both the conventional and API systems of identification. *X. campestris* does not appear in the Weaver scheme but the organism can be identified using conventional methodology and interpreting the results with the aid of Bergey's Manual. The API 20 E Profile also does not provide for the identification of *Xanthomonas* sp., so *X. campestris* ATCC 6402 was identified as a IIk-1. However, *X. campestris* is a rare isolate and, therefore, does not present a problem. As more of these organisms are isolated from clinical specimens it will be added to the API Profile.

Overall, the API 20 E identifications correlated with the tube agglutinations for 95% (39/41) of the organisms. In contrast, if the criteria for differentiating IIk-1 from IIk-2 as described by Weaver et al. (42) were strictly adhered to, there was only 78% correlation (32/41) between the conventional system and the serological results.

If weak positive urease reactions and/or growth of only a few colonies on MacConkey agar were considered as negative results, the conventional system agreed with the tube agglutinations for 97% (40/41) of the organisms.

Thus, our results indicate that the API 20 E system would be the method of choice for identifying the nonfermentative gram negative rods. Although there are some strains of IIk and other nonfermenters that are difficult to classify by either system, the API produced few questionable results. The ease of performance and shorter incubation times needed for the API are also well suited for a clinical laboratory. One major drawback of the API 20, however, is in the interpretation of the reactions. Since the tests are the same as those used in the conventional system, interpretation of slight color changes may still vary from individual to individual.

Kirby-Bauer susceptibility tests indicated that the IIk-2 strains were highly resistant to the antibiotics commonly used to treat gram positive infections, to ampicillin, and to the aminoglycosides which are used in *Pseudomonas* therapy (Table 9a, 9b, 9c). Tetracycline and chloramphenicol would appear to be the drugs of choice against IIk-2. The IIk-1 strains were also highly resistant to the antibiotics used to treat gram positives except for vancomycin (Table 9c, 9d). In contrast to the IIk-2 organisms, the

IIk-1 strains were highly sensitive to the aminoglycosides and carbenicillin. Tetracycline and chloramphenicol would also appear to be very effective in controlling IIk-1 infections. Since the IIk-1 and IIk-2 biotypes have distinctive susceptibility patterns, it is possible to distinguish between the two by use of antibiograms.

Antimicrobial susceptibility patterns can also be used to aid in the differentiation of Group IIk from other nonfermenters. For example, all strains of P. aeruginosa are sensitive to colistin (23). In contrast, all but two of the 41 strains of IIk tested in this study were resistant. Also, P. fluorescens, which is uniformly sensitive to gentamicin (23), could usually be differentiated from the IIk-2 strains since only 4% (1/24) of the strains were susceptible. This technique must be used with caution, however, since rapid changes in susceptibility patterns may occur due to mutations and episomal transfer of drug resistance (23).

When unheated IIk strains were tested against anti-IIk antisera most of the organisms tended to react with a large number of antisera, that is they were polyagglutinable. However, when the antisera were tested against heated IIk strains, the reactions were found to be relatively specific for the subgroups of the IIk bacteria. A similar phenomenon was reported to occur by Elrod and

Braun (14) when they performed tube agglutination studies on Xanthomonas. Apparently, a polysaccharide "gum" was responsible for these cross-reactions, and it was eliminated by growth on media without added carbohydrate followed by heat treatment.

The serological reactivity of the IIk organisms was also studied employing the indirect fluorescent antibody technique (Table 15). Minor differences in reactivity patterns were noted when the results of the tube agglutination and fluorescent assays were compared. In general, the titers of the antisera were slightly higher when tested by the indirect fluorescent antibody method. Also, a greater number of organisms showed apparent weak cross-reactivity by the fluorescent assay and this tended to obscure the presence of subgroups. Therefore, it was felt that use of the indirect fluorescent antibody technique would not aid significantly in the characterization of the IIk organisms and no further experiments were undertaken.

A panel of 17 antisera was selected which would, hopefully, detect all the IIk strains. An aliquot of each of the antisera was adsorbed with each organism exhibiting a positive agglutination in the tube tests. Heat-treated organisms were used for retesting the adsorbed antisera by tube agglutination in order to eliminate possible cross-reactivity due to heat-labile antigens.

In order to prepare antisera which were monospecific for IIk-1 O antigens, however, it was necessary to adsorb each anti-IIk-1 antiserum with a polyagglutinable organism, i.e. K 2. A similar phenomenon was reported by Duncan et al. (12) when they attempted to prepare O specific antisera for *P. aeruginosa*. They suggested that polyagglutinating strains may react with agglutinins formed against thermo-stable components, possible "core" polysaccharide. After adsorbing the anti-IIk-1 antisera with K 2, titers against the remaining cross-reacting organisms were generally elevated. To determine the serum factors that were involved in this phenomenon, an experiment was performed in which the material adsorbed to the surface of the bacterial cells was eluted with glycine HCl (pH 3.0), neutralized, electrophoresed for 1 h, and then tested against goat anti-normal rabbit serum. A single arc formed in the IgG region suggesting the existence of an antibody that partially blocked the specific O-anti-O reaction of the unadsorbed antiserum. Further investigations would be necessary to determine the nature of these components.

Cross-adsorption studies on the anti-IIk-1 antisera indicated the presence of 8 distinct subgroups (Tables 16-23). Only 2 strains, 76-933 and 76-942, did not agglutinate in any of the adsorbed antisera, and, therefore, could not be placed in any specific subgroup. It would be necessary

to prepare antisera against these two strains to determine whether they are members of one or more additional subgroups. Alternatively, these two organisms may actually belong to one of the defined serogroups but did not produce enough of their O antigens under the growth conditions utilized.

Adsorption of the anti-IIk-2 antisera with polyagglutinating strain 2147G was necessary to prepare monospecific anti-O antisera. A rise in the O specific titer was evident with only one antiserum, anti-C 198, when retested against the homologous organism (Table 27). The data, therefore, suggest the existence of agglutinins which reacted with heat-stable antigen in the IIk-2 strains which generally did not block the specific O-anti-O reaction. After adsorption with 2147G to remove the nonspecific cross-reacting antibodies, most (22/24) of the IIk-2 strains could be readily placed into one of the 9 subgroups based on their serological reactions. Only 2 out of the 24 strains, 76-945 and 74-1540, were not agglutinated by any of the adsorbed anti-IIk-2 antisera. Therefore, they could not be placed into a particular subgroup. Further studies would have to be done in order to determine whether one or more additional subgroups exist or if these strains were unable to produce adequate O antigen under the conditions utilized.

It was noted that unusual nonreciprocal cross-

reactivity patterns occurred in several instances when certain of the adsorbed antisera were retested with the apparent cross-reacting organisms. For example, adsorption of anti-1852G with 1852G IIk-1 removed the reaction of the antiserum when tested against P. fluorescens ATCC 13525 but adsorption of the same antiserum with P. fluorescens ATCC 13525 did not remove the reaction with 1852G (Table 21). In addition, when anti-2146G was adsorbed with 2146G IIk-2 the reaction of the antiserum with 2146G and LMC 3 was eliminated, but adsorption of anti-2146G with LMC 3 was not able to eliminate the reaction with 2146G (Table 28). Since adsorption of the antisera with the heterologous bacteria did not decrease any of the homologous titers, this phenomenon cannot be explained on the basis of a shared antigen. Dudman (11) observed a similar occurrence between strains of Rhizobium japonicum and suggested that either cross-reacting antibodies were involved, or the antigenic determinants were present but only in limited amounts. Therefore, 2146G and LMC 3 may actually be members of two different subgroups. Further studies would be necessary to clarify these points.

Since the tube agglutination studies revealed minimal cross-reactivity between the two IIk biotypes, and between Group IIk and other nonfermentative rods, serological techniques may be a useful method of differentiating the IIk organisms. Before this system could be used

in a clinical laboratory, however, it will be necessary to do further studies on larger numbers of organisms to better understand their immunological properties. Standardized strains and antisera could then be defined.

After cross-adsorption studies were completed and the O specific subgroups described, a correlation could be seen between the specific subgroups and their respective antibiotic susceptibility patterns. For example, all 7 members of IIk-2 subgroup I show similar sensitivity to tetracycline, chloramphenicol, and carbenicillin, and resistance to the remainder of the antibiotics tested (Table 9a). The antibiograms of the IIk-2 organisms in subgroups II, V, VII, and IX vary from subgroup to subgroup but are virtually identical within each subgroup (Table 9a, 9b, 9c). The 2 organisms in subgroup IV, 2146G and LMC 3, have different antibiotic susceptibility patterns which would be expected by examination of their serological reactions (Tables 9b, 28). Subgroup III, which contains only one organism, C 198, has a sensitivity pattern which differs from the other subgroups. Strains 76-945 and 76-1540 were not detected by any of our adsorbed antisera and also have different antibiograms suggesting that there may be one or more additional subgroups in the Group IIk-2 organisms.

A similar correlation can be seen between the antibiograms of the IIk-1 organisms and their O specific subgroups, but the variability within groups is somewhat

greater than for the IIk-2 bacteria. The antibiograms of subgroup VIII (Table 9d), for example, are identical; however, only 3 out of the 4 strains in subgroup I have similar susceptibility patterns (Table 9c). The remaining strain in subgroup I, 76-941, is serologically and biochemically identical to the other 3 members of the subgroup, but exhibited a significantly different sensitivity pattern. The organisms in subgroup IV show a similar discrepancy. Strains 1897G, LMC 1 and LMC 2 have similar antibiograms but 1897G alone is resistant to ampicillin. The members of subgroup II, 1672G and LMC 4, differ only in their susceptibility to penicillin (Table 9c). Subgroup III, which consists of K 2, 76-933, and 76-942, probably only share a common "core" antigen. Their sensitivity patterns differed markedly. Since subgroups V, VI, and VII are each composed of only one organism, it is impossible to make any correlation between serological subgroups and antibiograms at this time.

The reproducibility of Kirby-Bauer susceptibility testing is in the range of 90% confidence limits when compared to tube dilution minimal inhibitory concentration (MIC) results (21). Also many indeterminate zone sizes are actually sensitive when MIC's are determined. It is possible, therefore, that MIC experiments could eliminate the discrepancies observed in the susceptibility patterns within the IIk-1 subgroups.

Thus, analysis of the biochemical, serological, and antibiotic susceptibility patterns of the IIk strains indicates that differences in serological reactivity can be correlated with differences in antibiograms. Although more in depth studies are needed, this suggests that it may be possible to develop a simple agglutination procedure which would permit the rapid presumptive identification, and determination of the antibiograms of IIk strains isolated from clinical specimens.

SUMMARY

Biochemical, antimicrobial, and serological techniques were employed to study the nonfermentative gram negative rods in Groups IIk-1 and IIk-2 in order to develop a simple, reliable method for their identification. The API 20 Enteric system was used to identify strains of IIk-1 and IIk-2 and the results were compared to those obtained by the conventional biochemical method of Weaver. Antibiotic susceptibility patterns were determined utilizing the Kirby-Bauer single disk diffusion method. Antisera were also prepared against 14 strains each of IIk-1 and IIk-2 and against 5 ATCC control organisms in order to study their antigenic makeup.

When the results of the API 20 E system were compared to those obtained by the conventional method, they were found to be in agreement for 93% (38/41) of the IIk strains tested. Strict adherence to the criteria described by Weaver for the differentiation of the two biotypes of IIk, however, resulted in only 78% correlation between the conventional biochemical method of identification and the results obtained from tube agglutination studies. In contrast, the API 20 system showed 95% (39/41) correlation

with the serological data. Also, due to the ease of performance and the shorter incubation time involved, the API 20 E was considered to be superior to the conventional biochemical system of identification.

The antibiotic susceptibility patterns of the two biotypes of IIk were very distinctive. The IIk-2 strains were much more resistant to the antibiotics tested than were the strains of IIk-1, particularly to the aminoglycosides. Therefore, antibiograms also proved useful in distinguishing IIk-1 from IIk-2.

When tube agglutination tests with heat treated antigen were utilized very little cross-reactivity was observed between the biotypes of IIk or between the ATCC control organisms and the IIk strains. In order to prepare antisera monospecific for the O antigens, however, it was necessary to adsorb each antiserum with a polyagglutinable organism. Adsorption of the anti-IIk-1 antisera with polyagglutinating strain K 2 not only removed nonspecific agglutinins, but also often resulted in a rise in titer when the antisera were retested against the homologous strains. Adsorption of the anti-IIk-2 antisera with polyagglutinating strain 2147G resulted in the removal of nonspecific agglutinins but exhibited no change in the titer when reacted against the homologous organisms.

No one antiserum was capable of detecting all strains

in either of the Group IIk biotypes. Adsorption studies, in fact, revealed the existence of 8 serogroups among the IIk-1 strains and 9 serogroups among the strains of IIk-2. The data suggest, therefore, that with the development of clearly defined, standardized strains and antisera; serological techniques will be useful in the identification of the IIk organisms.

Following definition of the O specific subgroups of IIk, a correlation was observed between the organisms of a particular subgroup and their antibiograms. The antibiotic susceptibility patterns varied from subgroup to subgroup but were virtually identical within each subgroup. Therefore, it may be possible in the future to use a simple agglutination procedure for the rapid presumptive identification of the IIk organisms and, thereby, to also determine their susceptibility patterns.

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APPROVAL SHEET

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology.

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